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POLYPEPTIDES OF MURINE AND AVIAN PNEUMOVIRUSES

by ROGER LING (BSc, Warwick)

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Declaration

I hereby declare that all the work presented in this thesis is my own unless indicated otherwise in the text or in the Acknowledgements and has not been submitted for a degree at any other institution.

Dedication:-

1) To my parents

or

2) Something I have run out of!

Publication:-

LING R. & PRINGLE, C. R. (1988)

Turkey rhinotracheitis virus: *in vivo* and *in vitro*
polypeptide synthesis *Journal of General Virology* 69,
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Abbreviations

Ag8.653	X63-Ag8.653
BHK	Baby hamster kidney
CDV	Canine distemper virus
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Endo- α -N-acetylgalactosaminidase	O-Glycopeptide endo-D- galactosyl-N-acetyl- α - galactosamino hydrolase
Endo D	Endo- β -N-acetyl- glucosaminidase
Endo F	Endo- β -N-acetyl- glucosaminidase
Endo H	Endo- β -N-acetyl- glucosaminidase
EDTA	Ethylenediaminetetracetic acid, disodium salt
FACS	Fluorescent activated cell sorter
Glycopeptidase F	Glycopeptidase-N- glycosidase
M _r	Relative molecular mass
NEPHGE	Non-equilibrium pH gradient electrophoresis
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PMSF	Phenylmethanesulphonyl- fluoride
PEG	Polyethylene glycol
PHLS	Public health laboratory service
PVM	Pneumonia virus of mice
RNA	Ribonucleic acid
RS	Respiratory syncytial
SDS	Sodium dodecyl sulphate
TEMED	Tetramethylethylenediamine
TLCK	Tosylsulfonyl-lysyl chloromethylketone
TPCK	Tosylsulfonyl-phenylalanyl chloromethylketone
TRT	Turkey rhinotracheitis virus
VSV	Vesicular stomatitis virus

Abbreviations for tissue culture media and buffers and solutions are given in Sections 2.3 and 2.4 respectively

Summary

The work described in this thesis identifies some properties of the major polypeptides of pneumonia virus of mice (PVM) and of turkey rhinotracheitis (TRT) virus. The PVM glycoproteins have been studied in particular detail while the results obtained with TRT virus provide a preliminary description of the polypeptides of this virus. Twelve major PVM specific polypeptides designated L, G1, G2, F1, N, 39K, 35K, M, 20K, 19K, 16K and 12K were identified. In addition PVM specific polypeptides designated 25K, 24K, 23K, 18K and 17K were sometimes detected. Monoclonal antibodies directed against the G1/G2, 39K and M polypeptides were produced.

The ability of a monoclonal antibody to precipitate G1 and G2 suggested that these two glycosylated proteins were related and this was confirmed by tryptic peptide mapping. G2 was shown to be derived from G1 in pulse chase experiments and a similar relationship between two higher mobility polypeptides synthesized in the presence of tunicamycin was observed. The G protein may have a precursor since G1 did not appear immediately following a pulse labelling. The precursor could not however be identified. An additional minor glycosylated polypeptide of 42K was found to be related to the G protein.

The F1 protein appeared to be poorly glycosylated and a difference in mobility of the polypeptide synthesized in the presence of tunicamycin did not appear to be directly due to a lack of N-linked oligosaccharides. The polypeptide migrated more slowly under non-reducing conditions but no evidence of a small disulphide bonded polypeptide was found in contrast to the situation with other paramyxoviruses. This polypeptide appeared to be the major PVM protein expressed on the cell surface and was associated with G1 and G2 as the major protein in a particulate fraction of the infected cell supernatant.

Tentative relationships were suggested between the 39K, 35K and 25K polypeptides, the M and 24K polypeptides and the 20K and 19K polypeptides. This together with the observation that the 12K polypeptide was not a primary gene product suggested that there may be about 11 PVM polypeptides. The N or 39K and the 20K or 19K polypeptides were observed to be phosphorylated.

Twelve possible TRT virus specific polypeptides of 150K, 129K, 95K, 83K, 57K, 45K, 38K, 35K, 30K, 23K, 19K and 15K were identified. The 150K, 95K, 83K, 57K, 45K and 15K polypeptides were glycosylated with the latter three polypeptides showing a similar relationship to the F1,2, F1 and F2 polypeptides of paramyxoviruses. A broad glycosylated band designated the 31K polypeptide was identified that was similar to a smeared band observed on prolonged exposure of immunoprecipitates of PVM polypeptides labelled with [³H]-glucosamine. The 35K and 19K polypeptides were observed to be phosphorylated.

PVM may be more closely related to RS virus than TRT virus since anti-PVM serum immunoprecipitated the RS virus N polypeptide but not any TRT virus polypeptides. The PVM 39K polypeptide and the RS virus P protein were recognised by a monoclonal antibody providing further evidence of a relationship between PVM and RS virus.

INTRODUCTION

INTRODUCTION

The genus *Pneumovirus* is a group of viruses belonging to the family *Paramyxoviridae*, the other genera in this family being the genus *Paramyxovirus* which includes mumps virus and parainfluenza viruses 1, 2, and 3, and the genus *Morbillivirus*, which includes measles virus, canine distemper virus, rinderpest virus and peste des petits ruminants virus (Kingsbury et al., 1978). The *Paramyxoviridae* are classified in group V of the Baltimore classification, having a single stranded RNA genome complementary to the viral messenger RNA. They are enveloped viruses having glycoprotein spikes and an internal nucleocapsid structure containing the viral RNA. The genome consists of a single RNA molecule which is transcribed from a single promoter at the 3' end. The classification of the *Paramyxoviridae* into the three genera is based largely on the biological properties of the larger of the two glycoproteins and, in the case of the pneumoviruses on the dimensions of the nucleocapsid. The large glycoprotein of the paramyxoviruses, designated HN, is responsible for haemagglutination by the virus and its neuraminidase activity, in the morbilliviruses the analogous protein designated H has haemagglutinating activity only. Respiratory syncytial virus, the type member of the *Pneumovirus* genus lacks both activities. The genus *Pneumovirus* has 3 established members, human respiratory syncytial virus, bovine respiratory syncytial virus and

pneumonia virus of mice, (PVM). Recently a fourth virus which may be placed in this genus has been described, Turkey rhinotracheitis virus (TRT virus). The involvement of these viruses in disease of man and animals and a description of the genome structure and the virus associated polypeptides that have been observed are described below. One of the aims of the project was to compare the polypeptides of PVM with those of RS virus and so RS virus polypeptides are described in some detail. The introduction is concluded with the aims of the work described in this thesis.

1.1 Human respiratory syncytial virus

Human respiratory syncytial virus is a major cause of respiratory disease in young children, being associated with bronchiolitis and pneumonia in infants under 1 year of age more often than any other agent. The involvement of this virus in respiratory disease has been reviewed by Chanock et al. (1976) and Stott and Taylor (1985). The virus was first isolated from children with respiratory disease by Chanock et al. (1957) and was found to be antigenically related to an agent isolated earlier from chimpanzees showing mild upper respiratory tract disease (Morris et al., 1956). The name of the virus was given to it because of its cytopathic effect on cells in culture. The studies reviewed by Stott and Taylor (1985) and Chanock et al. (1976), showed that most infants were infected during the first year of life and that reinfection was common, most disease in infants being observed in the winter months in temperate climates. The

virus does not usually cause lower respiratory tract disease in adults although infection is rarely believed to be asymptomatic, usually causing upper respiratory tract infection. Infections are more severe in immunocompromised patients and the elderly. In old peoples homes and geriatric wards for example a disease clinically similar to influenza and commonly progressing to bronchopneumonia with significant mortality levels has been⁶ observed (Garvie & Gray, 1980; PHLS, 1983). Adult volunteers have been infected with respiratory syncytial virus despite having serum neutralizing antibody (McKay et al., personal communication; Mills et al., 1971) and in a comparison of consecutive natural infections, Beem (1967) failed to detect any difference between virus strains using sera from the infected individuals although differences were observed using ferret sera. In contrast monoclonal antibodies have been used to distinguish 2 (Mufson et al., 1985) or 3 (Anderson et al., 1985) subgroups of the virus but how these relate to the occurrence of reinfections is not clear.

The A and B subgroups of RS virus differ not only in reaction patterns with monoclonal antibodies but also in the mobilities of the F and P proteins (Gimenez et al., 1986; Norrby et al., 1986). The gene coding for the G protein, which showed the greatest number of differences between antigenic sites between the two subgroups (Mufson et al., 1985) has been shown to differ significantly in sequence between the two subgroups (Johnson et al., 1987a). Most of the data described in the following sections refer to A

subgroup viruses with which most of the published work was carried out.

1.1.1 Genomic organization of the A subgroup of Respiratory syncytial virus.

The order of the RS virus genes on the genome has been determined by comparison of size and sequence homology of polycistronic readthrough⁶ transcripts identified by hybridization of cDNA clones of the viral mRNA molecules with Northern blots of infected cell RNA, (Collins & Wertz, 1983; Collins et al., 1984a). The transcription of the genes from a single promoter and the order in which the transcripts are made have been demonstrated by Dickens et al. (1984) using ultraviolet inactivation studies. The two maps give the same gene order which is shown in Figure 1 along with the gene orders of other non-segmented negative stranded RNA viruses. The nucleocapsid gene N is the first gene to be transcribed in all of the viruses shown in Figure 1 except for RS virus where two small genes, NS1C and NS1B are located immediately after the promoter. Translation of viral messenger RNA molecules isolated by hybrid selection with the corresponding cDNA clones showed that NS1C and NS1B coded for polypeptides migrating in SDS-PAGE with mobilities corresponding to M_r s of 14,000 and 11,000 respectively (Collins et al., 1984a). The M_r s of these polypeptides obtained from sequence data are 15,565 and 14,674 (Collins & Wertz, 1985c). The messenger RNA molecules corresponding to these genes are the most abundant in

Figure 1 Gene order of non-segmented negative strand viruses

This figure, redrawn from Pringle (1987), shows the gene order of rhabdoviruses and paramyxoviruses. The 3' promoter (Pr) is shown at the top and the 5' end of the genome at the bottom. Genes present only in some viruses are shown in blue, genes encoding a fusion glycoprotein are shown in red and genes encoding an attachment glycoprotein are shown in orange. The original sources of the gene arrangements were as follows:-

VSV	Hudson et al. (1986)
Rabies virus (RV)	Tordo et al. (1986)
Haematopoietic necrosis virus (HNV)	Kurath et al. (1985)
Measles virus (MV)	Rima et al. (1986)
	Dowling et al. (1986)
Parainfluenzavirus type 1 (PI1)	Blumberg et al. (1985a,b)
	Shioda et al. (1986)
SV5/parainfluenzavirus type 2 (PI2)	Paterson et al. (1984)
Newcastle disease virus (NDV)	Chambers et al. (1986a)
Respiratory syncytial virus (RS)	Collins et al. (1984a,b)

<i>Rhabdoviridae</i>		<i>Paramyxoviridae</i>			
<i>Vesicular</i>	<i>Lysse</i>	<i>Morbili</i>	<i>Paramyxo</i>		<i>Pneumo</i>
<i>-virus</i>	<i>-virus</i>	<i>-virus</i>	<i>-virus</i>		<i>-virus</i>
VSV	HNV/RV	MV	PI1/PI3	SVS/NDV	RS
3'	3'	3'	3'	3'	3'
Pr	Pr	Pr	Pr	Pr	Pr
					NS1C
					NS1B
N	N	NP	NP	NP	N
NS/SORF	M1	P/C	P/C	P/C	P
M	M2	M	M	M	M
					NS1A
		F0	F0	F0	G
G	G			SH	
		H	HN	HN0	F0
	NV				M2
L	L	L	L	L	L
5'	5'	5'	5'	5'	5'

infected cells as expected from the predicted attenuation of transcription with increasing distance from the promoter (Collins & Wertz, 1983).

The gene order of the N, P, and M genes is similar to that observed for other paramyxoviruses and rhabdoviruses. The N gene of RS virus has an open reading frame encoding a protein of 391 amino acids with a calculated M_r of 42,600. The P gene encodes a polypeptide of M_r 27,150 in the A2 strain (Satake et al., 1984) and 27,166 in the Edinburgh strain (Lambden, 1985), both being devoid of cysteine and tryptophan residues. The M_r calculated from the sequence is less than that observed by SDS-PAGE. This may be due to the altered binding of SDS to proteins rich in aspartic acid and glutamic acid residues.

The P gene of the A2 strain of RS virus does not have a second open reading frame as has been observed for the corresponding gene in vesicular stomatitis virus, (Herman, 1986; Hudson et al., 1986), measles virus (Bellini et al., 1985) and Sendai virus, (Giorgi et al., 1983; Shioda et al., 1983) or an internal gene at a separate initiation site as observed in mumps virus (Herrler & Compans, 1982), Newcastle disease virus (Collins et al., 1982) and SV5 (Peluso et al., 1977; Paterson et al., 1984). Lambden (1985) has reported a second open reading frame in the Edinburgh strain of RS virus which overlaps the 3' end and potentially encodes a protein of 65 amino acids. The significance of this is not known.

The M gene of RS virus has an open reading frame

encoding a polypeptide of 256 amino acids with a calculated M_r of 28,717 (Satake & Venkatesan, 1984). The protein has two clusters of hydrophobic amino acids in the carboxy terminal third of the molecule which could interact with membrane components. A second open reading frame in the M gene could potentially encode a polypeptide of 75 amino acids but this has not been observed and the initiation codon, unlike that for the M protein does not lie in a conserved sequence found in functional eukaryotic initiation sites. Some recombinant plasmids with M gene sequences had been observed to select messenger RNA for M and a 15,000 M_r polypeptide but no suitable sites could be found in the RS virus sequences known that could act as donor sites for a spliced message encoding such a polypeptide.

RS virus has an additional gene located between the matrix protein gene and the glycoprotein genes compared with the other paramyxoviruses. This gene encodes a polypeptide with an M_r of 7536, designated NS1A (Collins & Wertz, 1985b), which has an apparent M_r of 9500 when it is synthesized *in vitro*, (Collins et al., 1984a). This polypeptide has two potential N-linked glycosylation sites and exists in infected and uninfected cells in both glycosylated and unglycosylated forms (Olmsted et al., personal communication).

The RS virus glycoprotein genes are arranged on the genome in a different order compared with those of other members of the *Paramyxoviridae*. The fusion protein gene of

RS virus has a similar structure to that of other members of the *Paramyxoviridae*, the location of cysteine residues in RS virus being similar to that in the Newcastle disease virus fusion protein (Chambers et al. 1986b). The F gene encodes a polypeptide of 574 amino acids with a calculated M_r of 63,453 for the A2 strain (Collins et al., 1984b) and 63,417 for the RSS-2 strain (Baybutt & Pringle, 1987). Collins et al. (1984b) identified an amino terminal signal sequence at residues 1 to 22, a transmembrane region at residues 525 to 550, a cleavage site at position 131 to 136 and a hydrophobic region at residues 137 to 154 which would become the amino terminus of the larger fragment, F1. The amino terminal F2 portion was relatively hydrophilic and had 4 of the 5 possible N-linked glycosylation sites. The amino terminal signal sequence showed less homology between strains than the remainder of the F gene sequence.

The G glycoprotein gene of RS virus differs significantly from the analogous gene of other non-segmented negative strand viruses. The M_r of the polypeptide coded for by the gene for the G glycoprotein is 32,587 compared with an estimated size for the glycoprotein in polyacrylamide gels of 84,000 to 90,000 (Wertz et al., 1985). The sequence contains a high proportion of serine and threonine residues which are potential acceptor sites for O-linked glycosylation, this type of modification being believed to account for most of the difference between the predicted size of the primary gene product (and that produced *in vitro*) and the estimated size of the polypeptide observed

on SDS-polyacrylamide gels. There is no amino terminal hydrophobic signal sequence or carboxy terminal hydrophobic membrane anchoring region. Wertz et al. (1985) suggest that a hydrophobic region near the amino terminus may perform both these functions.

The M2 (22K or 24K), protein gene of RS virus has no counterpart in other viruses in the family *Paramyxoviridae*. Viruses of the *Lyssavirus* genus of the *Rhabdoviridae* have a gene between the glycoprotein gene and the L protein gene (Tordo et al., 1986; Kurath et al., 1985). However this gene encodes a non-structural protein in infectious hematopoietic necrosis virus and is non-functional in rabies virus. In contrast the RS virus M2 protein gene encodes an envelope associated protein (Huang et al., 1984, 1985). The sequence of the M2 protein gene has been determined for the A2 strain by Collins & Wertz (1985a) and for the RSS-2 strain by Baybutt & Pringle (1987). The sequences suggest that it is a hydrophillic protein and is the most basic of the RS virus proteins. The protein coded for by this gene has 194 amino acids and a M_r of 22,153 for the A2 strain and 22,140 for the RSS-2 strain. A second open reading frame also exists in this gene which potentially encodes a protein of 90 amino acids, however no protein corresponding to this has been observed *in vivo* or *in vitro*.

Only a small part of the sequence of the L gene has been determined. This gene is presumed by analogy with the largest gene in VSV to encode the virion transcriptase/polymerase. The L gene was found to lack the 9

nucleotide start sequence that is conserved between all the other RS virus genes (Collins et al., 1987). The start of the L gene was found to overlap the end of the M2 gene and showed two nucleotide differences from the conserved sequence at the start of the gene. The most abundant L gene transcript was found to be a truncated transcript probably due to recognition of the M2 gene transcription termination and polyadenylation signals. Readthrough transcripts were known to occur for all the RS virus genes and this was proposed to be the mechanism for production of full length L gene transcripts. It is not known if the truncated L mRNA is simply a by-product of transcriptional attenuation or if it is also a functional mRNA since it contains two open reading frames (Collins et al. 1987).

Collins et al. (1986a) have sequenced the intergenic regions of RS virus and found no conserved features apart from a conserved adenosine at the end. The intergenic region between the F and M2 genes is less conserved than the sequences of the two genes between the A2 and RSS-2 strains (Baybutt & Pringle, 1987). Each gene had a sequence 5'-GGGGCAAU-3' at the beginning except for the presumed start of the L gene. The gene sequence ended with AGUUA(A/C)(A/U)(A/U)(A/U)AAAA for the 1C, N, P, M, 1A and G genes or AGU(A/U)(A/U)(A/U)AAAA for genes 1B, F and M2.

1.1.2 Polypeptides of human respiratory syncytial virus

The first experiments carried out to characterize the polypeptides of RS virus identified most of the structural

proteins of the virus but did not establish the virus specificity of the G glycoprotein (Cash et al., 1977; Cash et al., 1979b; Levine, 1977; Peeples & Levine, 1979; Wunner & Pringle, 1976). These authors identified polypeptides corresponding to the L, F1, N, P, M and M2 polypeptides. Levine (1977) and Peeples & Levine (1979) identified the G protein in the Long strain of RS virus purified from the supernatants of Hela cells infected at high multiplicity. They could detect the G polypeptide using either [³H]-glucosamine or a ³H amino acid mixture to label the polypeptides. Cash et al. (1977,1979b) and Wunner & Pringle failed to identify this protein using [³⁵S]-methionine labelling of RS virus polypeptides in BS-C-1 cells infected at a low m.o.i with several strains of RS virus. A polypeptide with a mobility corresponding to that of the G protein was identified using [³H]-glucosamine labelling but there was a cellular glycoprotein with the same mobility. It has subsequently been found that the virus strain, cell type and label used affect the ease with which the G glycoprotein can be identified (Dubovi, 1982; Pringle et al., 1981; Routledge et al., 1986).

The non-structural polypeptides corresponding to the products of the NS1A, NS1B and NS1C genes have been observed less often partly because many studies concerned the polypeptides of partially purified virus. Cash et al. (1977) detected a 10K polypeptide that could have corresponded to the 9.5K or 11K polypeptide products of the NS1A or NS1B genes. Dubovi (1982) detected a 14K polypeptide that could

have corresponded to the product of the NS1C gene. Cash et al. (1979b) detected a 17K polypeptide amongst the polypeptides synthesized *in vitro* using RNA from RS virus infected cells, the nature of which is not clear. The products of the NS1A, NS1B and NS1C genes have been observed in *in vitro* translation experiments (Collins et al., 1984a). The properties of the RS virus polypeptides that have been described are given below.

The L polypeptide

The L polypeptide is not well labelled probably due to attenuation of transcription and production of truncated mRNA molecules as described above in Section 1.1.1. It has been identified both in infected cells (Cash et al., 1977) and in purified virus (Peeples & Levine, 1979). Detergent and salt treatment of purified virus (Peeples & Levine, 1979), purification of nucleocapsids from virus on caesium chloride gradients (Lambert & Pons, 1983) and isolation of nucleocapsids by gel filtration of clarified cell lysates (Huang et al., 1984) have revealed that the L protein is nucleocapsid associated. By analogy with the large polypeptide of the rhabdovirus VSV it has been suggested that this polypeptide is a component of the viral RNA dependent RNA polymerase. An RNA dependent RNA polymerase activity has been demonstrated for RS virus *in vitro* but the polypeptides associated with the reaction mixtures were not described (Mbuy & Rochovansky, 1984).

The G glycoprotein

The viral specificity of this polypeptide was not at first clear as described above. Pringle *et al.* (1981) showed that this polypeptide was not readily labelled with [³⁵S]-methionine but could be readily labelled with [³H]-glucosamine. Dubovi (1982) also observed that the G protein was not well labelled with [³⁵S]-methionine but could be labelled with [³H]-leucine. This lead to the conclusion that the G protein had a low methionine content which was confirmed by the sequence of the gene (Wertz *et al.*, 1985). Pringle *et al.* (1981) also observed that the intensity of the band representing the mature form of the G protein (M_r of 80,000 to 90,000), varied depending on virus strain and cell type used. In partially purified virus G was detected more readily with the Long and A2 strains than with the RSN-2 strain and was labelled better when the virus was grown in HEp-2 cells than when it was grown in BS-C-1 cells. Routledge *et al.* (1986) using monoclonal antibodies to the G protein in immunoprecipitation experiments observed two G related polypeptides of 50K and 45K as major bands in A2 strain infected BS-C-1 and Vero cells. These bands were not so prominent with the Long strain and were weak in Hela cells and HEp-2 cells. Routledge *et al.* (1986) did not observe any progression of label between these G related polypeptides in a pulse chase experiment so the relationship between these forms of the G protein is not known.

The nature of the oligosaccharides and the processing of precursors to give the mature form of the G protein has

been described by several authors. The effects of the inhibitors and glycosidases used to study virus glycoproteins are described in Appendix 1. The size of the unglycosylated polypeptide synthesized *in vitro* is only about 36K when determined by SDS-PAGE compared with the size of the mature glycosylated form of 80-90K (Wertz et al., 1985). Fernie et al. (1985) have proposed two possible explanations for this. The G protein may consist of a dimer of a glycosylated 45K polypeptide or the polypeptide may be heavily glycosylated. The latter explanation is considered the most likely by most authors. The nature of the oligosaccharides has been studied by the use of glycosylation inhibitors and digestion with endoglycosidases. Two types of oligosaccharides have been described for the G protein. Fernie et al. (1985), Gruber & Levine (1985a), Lambert & Pons (1984) and Wertz et al. (1985) showed that treatment of RS virus infected cells with tunicamycin resulted in the production of a G protein with a slightly increased mobility due to the absence of N-linked carbohydrate. It was observed that the G protein was labelled with [³H]-glucosamine under these conditions indicating that not all of the oligosaccharides of the G protein were N-linked. The N-linked carbohydrate of the mature form of G was not sensitive to endo-H showing that they were of the complex type (Fernie et al., 1985; Gruber & Levine, 1985a; Lambert & Pons, 1984). The susceptibility of the G protein to digestion with endo-D depended on the cell type in which the virus was grown. When CV-1 cells were used

an increase in mobility of the G protein was observed but with HEP-2 cells digestion with neuraminidase as well as endo-D was required to produce a mobility change (Lambert & Pons, 1984). Neuraminidase also enhanced the action of endo-D on the G protein of RS virus grown in CV-1 cells. This suggests that the presence of neuraminic acid in the glycoprotein was cell type specific and inhibited the action of endo-D which only cleaves some types of complex N-linked oligosaccharides. Endo-F resulted in the disappearance of a band corresponding to the mature G protein and the appearance of a 43K polypeptide of unknown relationship to the G or F protein (Lambert & Pons, 1984). The result of the digestions with endo-F has not been studied further and the authors did not offer an explanation of the result.

The presence of O-linked oligosaccharides on the G glycoprotein has been studied indirectly using monensin treatment of infected cells to inhibit Golgi apparatus functions. Gruber & Levine (1985a) and Fernie *et al.* (1985) observed a marked increase in the mobility of the G protein in monensin treated cell lysates. This together with the high serine/threonine content of the predicted amino acid sequence (Wertz *et al.*, 1985) has been interpreted as evidence for a high degree of O-linked glycosylation. The size of the G related protein in monensin treated RS virus infected cells (45K) suggests that a large part of the mobility difference between the size of the G protein calculated from the predicted amino acid sequence and that calculated from the results of SDS-PAGE analysis of the

protein is due to O-linked oligosaccharides. The relatively low incorporation of mannose into the G protein described by Gruber & Levine (1985a) is also consistent with a large proportion of O-linked oligosaccharides which usually lack mannose (Kornfield & Kornfield, 1980).

Synthesis of the G protein in RS virus infected cells has been studied in pulse chase experiments by Fernie et al. (1985) and Gruber & Levine (1985b). These authors observed a precursor of about 45K which decreased in intensity after chase periods whereas the G protein increased in intensity with increasing length of the chase periods up to about 50 minutes. Fernie et al. (1985) also observed a 29K polypeptide with [^3H]-leucine labelling that decreased in intensity with increasing length of the chase period. Labelling with [^3H]-glucosamine revealed the polypeptides described above and several additional polypeptides with mobilities intermediate between those of the 29K and 45K polypeptides. Monensin was observed to slow down the processing of the 45K polypeptide to give the fully glycosylated G protein which could only be observed with carbohydrate labels. The 45K polypeptide could be identified using ^3H -leucine label. Labelling with [^3H]-glucosamine or [^3H]-mannose (but not [^3H]-fucose or [^3H]leucine) revealed a ladder of glycosylated polypeptides from 26K to 45K that was more evident in samples from monensin treated cells than in samples from untreated cells. Unlike the mature G protein the higher mobility polypeptides were endo-H sensitive showing that they were not breakdown products of G that were

produced during the sample preparation. These polypeptides therefore appeared to represent precursors to G that had high mannose N-linked oligosaccharides but not complex or O-linked oligosaccharides. Tunicamycin did not inhibit the incorporation of [³H]-glucosamine or [³H]-fucose into G but did inhibit incorporation of [³H]-mannose which was consistent with the inhibition of addition of N-linked but not O-linked oligosaccharides to the G protein. A 70K polypeptide and a 34K polypeptide that chased out in tunicamycin treated infected cells probably represented the G and 45K polypeptides without their N-linked oligosaccharides. These results suggested that addition of O-linked oligosaccharides occurred more slowly and independently of addition of N-linked oligosaccharides.

Hendricks et al. (1987) found that the G protein had a calculated M_r of 82K in the supernatant compared with 88K when it was immunoprecipitated from cell lysates. These authors found that the G protein was the glycoprotein most readily precipitated from the supernatants of infected cells and could be detected at the top of a gradient used to separate virus from free polypeptides. They suggested that the G protein was released into the supernatant like the G_s protein of VSV. (The VSV G_s protein is a truncated form of the viral G protein that is synthesized intracellularly and secreted into the medium (Graeve et al., 1986; Chen et al., 1987).)

Antibodies directed against the G glycoprotein have been found to neutralize plaque formation by RS virus but

not to inhibit fusion of RS virus infected cells. Many anti-G monoclonal antibodies do not neutralize the virus. For example Fernie et al. (1982) found that none of 15 anti-G protein monoclonal antibodies neutralized the virus. Walsh & Hruska, (1983) found that one of two anti-G protein monoclonal antibodies neutralized RS virus in the presence of complement. Tsutsumi et al. (1987) suggested that the type of anti-G monoclonal antibodies produced depended on the immunization protocol used. When intraperitoneal injections with Freund's adjuvant were used the anti-G monoclonal antibodies did not neutralize. When *Bordetella pertussis* was used as the adjuvant the monoclonal antibodies obtained neutralized RS virus although some of them required the presence of complement to have this effect. When intranasal infection was used all the anti-G monoclonal antibodies obtained neutralized RS virus in the absence of complement. Three sites were identified on the G protein one eliciting production of antibodies that did not neutralize and two that elicited production of antibodies that neutralized the virus in the absence of complement. One of the two sites eliciting production of neutralizing antibodies was also present on the 50K G-related polypeptide. The overall results of the experiments described by these authors, in contrast to all other groups, showed that a higher proportion of anti-G protein monoclonal antibodies than anti-F protein monoclonal antibodies neutralized the virus. Monoclonal antibodies are not ideal for determining which proteins induce production of

antibodies inhibiting biological properties such as infectivity and cell fusion. This is because each monoclonal antibody only binds to a single antigenic site which may or may not be involved in the function being studied. Experiments with polyclonal antisera therefore give a better overall idea of the functions of the RS virus proteins.

Walsh et al. (1984a) raised antibody to the G protein purified by affinity chromatography using an anti-G protein monoclonal antibody. This serum neutralized the virus in the absence of complement but did not inhibit fusion. This serum also inhibited the binding of purified G protein to HEp-2 cells. This serum and an antiserum raised against the RS virus G protein expressed from a clone of the gene in a vaccinia virus vector inhibited the attachment of purified [^{35}S]-methionine labelled RS virus to HeLa cells (Levine et al., 1987). These results suggested that the G protein was the attachment protein of RS virus.

The F glycoprotein

The F glycoprotein of RS virus is a glycosylated polypeptide of about 70K that is cleaved to give two disulphide bond linked polypeptides of about 45-50K and 20-25K (Gruber & Levine, 1983; Lambert & Pons, 1983). The larger fragment (F1) can be readily labelled with [^{35}S]-methionine (Wunner & Pringle, 1976; Cash et al., 1977). A 140K dimer has also been identified under non-reducing conditions (Lambert & Pons, 1983; Walsh et al., 1985; Trudel et al., 1986). The F0, F1 and F2 related

polypeptides were observed to be basic (Dubovi, 1982). A rabbit antiserum directed against a preparation of RS virus spikes immunoprecipitated all of the F related polypeptides as well as the G protein. Walsh & Hruska (1983) observed a speckled immunofluorescence pattern using an anti-F monoclonal antibody compared with filament associated fluorescence with an anti-G monoclonal antibody suggesting a different distribution of the polypeptides in infected cells. F2 was observed to show a variation in mobility between cell types and to sometimes show two bands on SDS-polyacrylamide gels (Lambert & Pons, 1983, 1984). A fragment of the F1 polypeptide may also exist since Samson et al. (1986) obtained evidence of a polypeptide of about 20K being recognized by a monoclonal antibody that bound to F1. Samson et al. (1986) suggested that the 20K polypeptide was F2 since it was not observed under non-reducing conditions. However Norrby et al. (1986) observed a fragment of F1 which was also not observed under non-reducing conditions. This suggests that the 20K polypeptide identified by Samson et al. (1986) may have also been a fragment of F1 explaining its identification with an anti-F1 monoclonal antibody. Hendricks et al. (1987) observed a polypeptide of 18K that appeared to be related to the F protein in addition to F2. The nature of the various minor F related polypeptides is not understood but they are presumed to be breakdown products of F1 or F2.

Tunicamycin has been shown to inhibit glycosylation of the F protein suggesting that all of the oligosaccharides

were N-linked (Fernie et al., 1985; Gruber & Levine, 1985a). A change in migration of F1 and F2 after digestion with endo-D was observed similar to that with the G protein, which was dependent upon the absence of neuraminic acid residues as described for the G protein (Lambert & Pons, 1984). The mature F polypeptides were insensitive to endo-H (Gruber & Levine, 1985a). These results suggested that the N-linked carbohydrate was of the complex type. Endo-F treatment of samples led to an increase in mobility of F0 and a decreased mobility of F1 (Lambert & Pons, 1984) although since F specific antibodies were not used the identification of the F specific polypeptides was not necessarily correct.

F0 has been observed to be the precursor to F1 and F2 in pulse chase experiments (Fernie et al., 1985; Gruber & Levine, 1985b). A slight increase in the mobility of F1 and F2 labelled in RS virus infected cells incubated in the presence of monensin was observed (Gruber & Levine, 1985a). This effect is probably due to inhibition of some of the later stages of processing of the N-linked oligosaccharides in the Golgi apparatus. Monensin treatment of RS virus infected cells also caused a partial inhibition of the cleavage of F0 (Fernie et al., 1985). This suggested that cleavage of F0 occurs in the Golgi apparatus or at a later stage during transport to the cell surface. Unlike the F1 and F2 polypeptides F0 was sensitive to digestion by endo-H (Gruber & Levine, 1985a). Gruber & Levine (1985b) exposed monensin treated, RS virus infected cells to trypsin in

order to remove any F protein expressed on the cell surface and immunoprecipitated the cell lysates with anti-F protein serum. F1 and F2 were still detected and it was therefore concluded that cleavage occurred intracellularly.

Antibodies to the F protein have been used to show that it is involved in fusion of RS virus infected cells and that it is a target antigen for neutralizing antibodies. Walsh et al. (1985) raised antisera to F protein that had been purified by affinity chromatography. When antiserum was raised against the native polypeptide it was found to neutralize the virus but not to inhibit fusion. In contrast antiserum raised against the reduced polypeptide both neutralized the virus and inhibited fusion. The latter antiserum had higher levels of antibody recognizing the F2 protein. Antibodies to F2 are not essential to get a fusion inhibiting reaction however because monoclonal antibodies to F1 will inhibit fusion (Trudel et al., 1987a). Several groups have carried out solid phase competitive binding studies to identify antigenic sites on the F protein (Anderson et al., 1986; Fernie et al., 1982; Gimenez et al., 1986; Mufson et al., 1985; Norrby et al., 1986; Örvell et al., 1987; Routledge et al., 1986; Samson et al., 1986; Trudel et al., 1986; Trudel et al., 1987a; Tsutsumi et al., 1987; Walsh & Hruska, 1983; Walsh et al., 1986). Since these authors used different sets of monoclonal antibodies it is not possible to determine the total number of sites that have been described. Five sites bind antibodies inhibiting fusion and neutralizing the virus. Two sites bind

neutralizing antibodies that do not inhibit fusion and a further twelve sites bind neutralizing monoclonal antibodies whose reactions in fusion inhibition assays have not been reported. Three sites bound antibodies that neither inhibited fusion or neutralized the virus. Sixteen sites bound non-neutralizing antibodies whose reactions in fusion inhibition assays have not been reported. It appears likely therefore that only a small proportion of antigenic sites are recognized by neutralizing antibodies and a subset of these are recognized by fusion inhibiting antibodies. One site recognized by fusion inhibiting and neutralizing monoclonal antibodies has been found to be resistant to denaturation and at least part of the site has been localized to a 12 amino acid peptide (Trudel et al., 1987a,b). This is therefore a possible candidate for a synthetic peptide vaccine. The relevance of solid phase binding studies to the situation with native virus particles is not clear since Walsh et al. (1986) observed that a polyclonal antiserum inhibited binding of a fusion inhibiting monoclonal antibody in solid phase binding studies but did not inhibit the fusion inhibiting activity of the monoclonal antibody on RS virus infected cells in tissue culture. Antisera raised against purified F protein or vaccinia virus expressing the RS virus F protein did not inhibit attachment of RS virus to Hela cells in contrast to sera raised against the G glycoprotein suggesting that the F protein was not involved in attachment of the virus to cells (Levine et al., 1987).

The N protein

The N protein migrates on SDS-polyacrylamide gels with an estimated M_r of 40K to 44K. It has been identified as the major nucleocapsid polypeptide (Huang et al., 1984; Peeples & Levine, 1979; Wunner & Pringle, 1976). Cash et al. (1979b) identified a 38K polypeptide that was related to N. The 38K polypeptide was not identified in *in vitro* translation experiments but was more readily labelled than N in pulse labelling experiments carried out with infected cells. After a chase period N became more abundant than the 38K polypeptide. The results of experiments with TPCK and/or TLCK added during labelling or just during harvesting or storage suggested that the 38K polypeptide was derived from the N polypeptide by proteolytic cleavage. The explanation of these results is not clear. Cash et al. (1979b) suggested that the N protein was synthesized as a protease sensitive form and that later a protease resistant form appeared. This explanation does not appear to explain the apparent conversion of a 38K polypeptide into N during pulse chase experiments.

Fernie et al. (1981) found that a nucleocapsid associated polypeptide was expressed on the surface of Balb/c cells persistently infected with RS virus, although it does not normally appear on the surface of virus infected cells (Walsh & Hruska, 1983). The N protein does, however appear to be a target for cytotoxic T cells (Bangham et al., 1986; Pemberton et al., 1987) and so at least part of the protein may sometimes be expressed on the cell surface. No

monoclonal antibodies or anti-nucleocapsid sera have been found that neutralize RS virus. Hendricks *et al.* (1987) found that the N protein appeared to be released into the supernatant of infected cell cultures. The cells were claimed to be intact since the F polypeptide was not released into the supernatant but this may not be the case because the N protein may have been soluble whereas the F protein may have been membrane associated and not released from disrupted cells. The N protein is not readily resolved on the basis of charge in 2 dimensional non-equilibrium pH gradient electrophoresis/SDS-PAGE possibly due to aggregation or association with RNA (Dubovi, 1982). The N polypeptide has not been observed to be glycosylated phosphorylated or sulphated.

The P protein

The P protein migrates more slowly when studied by SDS-PAGE than would be expected from its M_r calculated from the predicted sequence. It has a M_r calculated from its mobility in SDS-PAGE of 31K to 35K depending on the strain compared with a M_r calculated from the sequence of 27K. It shows the most marked inter-strain variation in mobility on SDS-polyacrylamide gels of all the RS virus polypeptides (Cash *et al.*, 1977). It is called the phosphoprotein of RS virus because it is the most extensively phosphorylated of the RS virus polypeptides (Cash *et al.*, 1979b; Lambert & Pons, 1983). It has been observed to be an acidic protein as would be expected of a phosphorylated protein rich in acidic

residues (Dubovi, 1982). Virus fractionation studies suggest that the P protein is more closely associated with the nucleocapsid of the virus than the M protein (Peeples & Levine, 1979; Huang *et al.*, 1984). Lambert & Pons (1983) and Huang *et al.* (1984) observed that P was associated with nucleocapsids purified on caesium chloride gradients or by gel filtration respectively. Monoclonal antibodies to the P protein frequently co-precipitate the N protein suggesting that the two proteins are closely associated (Gimenez *et al.*, 1984). Immunofluorescence studies with monoclonal antibodies directed against the P protein gave differing results perhaps indicating differences in conformation of this protein at different locations in the cell (Gimenez *et al.*, 1984). Three monoclonal antibodies directed against the P protein gave cytoplasmic immunofluorescence whereas two other anti-P protein monoclonal antibodies gave only cell surface staining. One of the antibodies giving cytoplasmic staining did give fluorescence at the periphery of RS virus infected cells fixed at a late stage of infection.

The M protein

The M protein of RS virus has a mobility on SDS-polyacrylamide gels corresponding to a polypeptide with an M_r of about 26-28K. It is solubilized from virions by non-ionic detergent (Peeples and Levine, 1979; Huang *et al.*, 1984). The M protein has been identified at a peripheral location in acetone fixed RS virus infected cells using immunofluorescence with an anti-M protein monoclonal

antibody but it was not detected on the surface of unfixed cells (Routledge et al. 1987b). These results suggest that the M protein is a non-glycosylated matrix-like protein similar to that of paramyxoviruses, morbilliviruses and rhabdoviruses. Wunner & Pringle (1976) showed that the RS virus M protein behaved in a similar manner to the VSV M protein on gradients of metrizamide containing non-ionic detergent. Lambert & Pons (1983) identified the M protein in a preparation of nucleocapsids purified on a caesium chloride gradient but other groups have not found the M protein to be nucleocapsid associated (Wunner & Pringle, 1976; Huang et al., 1984). A RS virus specific protein that was probably M (although reported as having the rather high M_r of 33K) was observed to be basic (Dubovi, 1982). Cash et al. (1979b) and Lambert & Pons (1983) did not identify any phosphorylated polypeptide with a mobility corresponding to that of the M protein. Lambert et al. (1988) did however identify a phosphorylated polypeptide that they believed to be the M protein. Gruber & Levine (1983) observed two bands with mobilities similar to the M protein when radiolabelled viral proteins were analysed under non-reducing conditions and suggested that this polypeptide existed as two forms differing in the number or position of intra-molecular disulphide bonds. No anti-M protein monoclonal antibodies have been reported to have neutralizing activity.

The M2 protein

The M2 protein is a second non-glycosylated membrane

associated protein that occurs in RS virus but apparently does not have any counterpart in paramyxoviruses, morbilliviruses or rhabdoviruses (Huang et al., 1984; Routledge et al., 1987b). It has been observed to be a basic polypeptide (Dubovi, 1982). Like the M protein it is solublized by treatment of virions with non-ionic detergent (Huang et al., 1984). It has not been identified in preparations of nucleocapsids (Wunner & Pringle, 1976; Lambert & Pons, 1983; Huang et al., 1984). The M2 protein exists in three different forms when analysed by SDS-PAGE under reducing conditions and shows 5 different forms under non-reducing conditions (Routledge et al., 1987a). Six different forms of M2 appeared to exist under non-reducing conditions but two of these co-migrated. The six bands observed under non-reducing conditions corresponded to two forms of each of the bands observed under reducing conditions. The reason for the existence of different forms of the M2 protein when it was analysed under reducing conditions is not clear, but Routledge et al. (1987a) suggested that the bands were artefacts that arose during sample preparation. Different forms of the M2 polypeptide observed under non-reducing conditions were considered to be due to different numbers or arrangements of disulphide bonds. Unlike the M protein the M2 protein is expressed on the surface of infected cells late in infection (Routledge et al. 1987b).

The 14K (1C) polypeptide

The 14K polypeptide was not described in many early reports of RS virus polypeptides probably due to it being insufficiently labelled in cell lysate preparations and absent from purified virus. Dubovi (1982) described a 15K acidic polypeptide that probably corresponded to this polypeptide. The 14K polypeptide has been identified primarily in *in vitro* translation experiments. It was first described clearly as one of 3 proteins translated *in vitro* from a set of closely migrating mRNA molecules excised from a gel (Huang & Wertz, 1983). Collins *et al.* (1984a) used hybridization selection with cDNA clones to show that the 14K polypeptide was translated *in vitro* from a mRNA designated 1c from which it derives its other name of the 1C polypeptide. The same paper also clearly showed the presence of the 14K polypeptide in infected cell lysates and showed that partial digestion of the polypeptides with *S. aureus* V8 protease gave similar peptides. The 14K polypeptide is considered to be a non-structural protein since it has not been identified in preparations of purified virus. Nothing is known of its function.

The 11K (1B) polypeptide

The 11K polypeptide like the 14K polypeptide described above was first clearly identified by *in vitro* translation of mRNA eluted from a gel (Huang & Wertz, 1983). Cash *et al.* (1977) observed a 10K polypeptide in infected cell lysates which could have corresponded to this polypeptide or the

9.5K polypeptide. However the 10K polypeptide was not identified when the cells were labelled and the samples harvested in the presence of TLCK or TPCK to inhibit proteases suggesting that it may have been a breakdown product of a larger polypeptide (Cash et al., 1979b). The 11K polypeptide was first clearly identified in infected cell lysates by Collins et al. (1984a) who also showed that it gave a similar peptide pattern to the 11K polypeptide synthesized *in vitro* following digestion with *S. aureus* V8 protease. It is translated from the mRNA designated 1B giving it the alternative name of the 1B polypeptide. The 11K polypeptide is considered to be a non-structural polypeptide since it has not been identified in purified virions. The function of the 11K polypeptide is not known.

The 9.5K (1A) polypeptide

The 9.5K polypeptide, like the 14K and 11K polypeptides was first clearly identified by *in vitro* translation of mRNA eluted from a gel. It has been identified in cell lysates by Collins et al. (1984a) although they could not show that it was related to the polypeptide synthesized *in vitro* because it was too small to give more than one band in partial proteolysis experiments. Like the 14K and 11K polypeptides it was considered to be a non-structural polypeptide. However immunoprecipitation of RS virus infected cell lysates with an antiserum directed against a synthetic peptide corresponding to the predicted carboxy-terminal sequence of the 9.5K protein identified

glycosylated forms of the 9.5K polypeptide. A discrete band and a lower mobility broad smear of label centred at a mobility corresponding to a M_r of about 30K was observed (R. Olmsted, C. Caravokyri, personal communications). This type of glycosylated polypeptide is not unique to RS virus, the influenza B virus NB protein producing a similar broad smear on the gel which Williams & Lamb (1986) suggested was due to interactions of the carbohydrate to produce aggregates of the NB protein alone or with host cell proteins. It is not clear if the glycosylated form of the 9.5K polypeptide occurs in virions. A weak broad smear appears to be present in [^3H]-glucosamine labelled virus shown in the paper by Lambert & Pons (1983) although the authors did not consider this label to be associated with a viral protein.

1.1.3 Differences between polypeptides of the two subgroups of RS virus

Most of the information on RS virus described above refers to the A subgroup since the two most commonly used strains of RS virus, the A2 and Long strains both belong to this subgroup. The results of Cash *et al.* (1977, 1979b), Pringle *et al.* (1981) and Gimenez *et al.* (1984) referred to the RSN-2 strain of the B subgroup. The G protein has been observed to exhibit the greatest degree of variation in mobility when analysed by SDS-PAGE. This mobility variation is strain specific and not related to the subgroup of the virus (Norrby *et al.*, 1986; Routledge *et al.*, 1986). Variation in the mobility of the M2 protein is similarly not

dependent on the subgroup of the virus (Norrby et al., 1987). The P, F1 and F2 proteins do show subgroup specific differences in mobility (Table 6). All of these mobility differences are quite small relative to differences in mobility between polypeptides of RS virus and PVM (Cash et al., 1977). Other differences observed between the polypeptides of viruses of the two subgroups are the presence of an additional F related polypeptide with a mobility slightly lower than F2 in subgroup A viruses and the instability of glycoproteins of subgroup B viruses in tunicamycin treated cells.

1.2 Bovine and caprine respiratory syncytial viruses

Since the bovine and caprine RS viruses have not been distinguished in reaction patterns with monoclonal antibodies (Örvell et al., 1987) and little work has been published on the caprine RS virus these viruses are described together. Less detailed work has been reported at the molecular level on these viruses than on human RS virus so they are only considered briefly. Bovine RS virus was isolated from calves with respiratory disease (Paccaud & Jacquier, 1970) and has been shown to be important in lower respiratory tract infection in cattle (Inaba et al., 1972; Rossi & Kiesel, 1974). Caprine RS virus has been isolated from a pygmy goat with respiratory disease (Lehmkuhl et al., 1980) but little is known about the incidence of infection of goats with RS virus.

Bovine respiratory syncytial virus shows a close

antigenic relationship to human respiratory syncytial virus (Paccaud & Jacquier, 1970; Rossi & Kiesel, 1974). The viruses differ in the cell types that can be infected in tissue culture in that bovine RS virus will not grow in primate cell lines unlike RS virus (Cash et al., 1977). The polypeptide profile of bovine RS virus is essentially identical to that of human RS virus (Cash et al., 1977).

1.3 Pneumonia virus of mice

Pneumonia virus of mice (PVM) was first isolated from 3.5 to 4 week old albino swiss mice (Horsfall & Hahn, 1939, 1940). Mice were inoculated intranasally with nasopharyngeal washings obtained from patients with respiratory tract diseases other than influenza. Lung homogenates were then serially passaged by the intranasal route of infection. This procedure resulted in the appearance of transmissible pulmonary consolidations after 2 to 7 passages with deaths occurring at the earliest after 3 passages and on average after 5 passages. It was found that the same result was obtained when lung homogenates of normal mice were used and the infectivity therefore appeared to be due to an endogenous infectious agent. The susceptibility of mice to infection was found to vary depending on the supplier of the mice. One ten thousandth of the dose required to kill the mice, given intranasally, was sufficient to protect mice subsequently challenged with a lethal dose of virus. Two intraperitoneal injections of the virus were found to protect mice against challenge with any strain of the virus

but not against influenza virus infection suggesting that there was little antigenic variation in the virus. Antibodies to PVM appear to be common in mice (Horsfall & Hahn, 1940; Jakubik & Skoda, 1974). PVM was found to be strictly pneumotropic, not causing any infection unless inoculated by the intranasal route. It was thought to be pathogenic only in mice. Later Horsfall & Curnen (1946) found that PVM would cause pneumonia in cotton rats and hamsters. The infection was not contagious, uninfected mice kept in the same cages as infected mice did not become infected (Horsfall & Hahn, 1940; Smith et al., 1984). Passage of PVM in chick embryo cultures or BHK-21 cells resulted in a reduction in virulence in mice (Horsfall & Hahn, 1940; Harter & Choppin, 1967).

Pearson & Eaton (1940) identified an agent antigenically related to PVM from Syrian hamsters and Eaton & van Herick (1944) described a similar agent in cotton rats.

1.3.1 Serological evidence for the possible exposure of many mammals to a PVM like virus.

Eaton & van Herick (1944) found evidence of neutralizing antibodies to PVM in rabbits. Horsfall & Curnen (1946) studied sera from a variety of animals and showed that neutralizing antibody against PVM could be found in rabbits, cotton rats, monkeys (*Macaca mulatta*), man, chimpanzees, hamsters guinea pigs and mice. Neutralizing activity was absent from the sera of chickens and mongooses.

Gannon & Carthew (1980) found neutralizing activity against PVM in mouse and rat colonies but not in guinea pig colonies but the number of guinea pig colonies studied was small. Carthew et al. (1978) did obtain serological evidence of PVM infection in a guinea pig. Anti-PVM antibodies have also been detected in rats using an enzyme linked immunosorbent assay (ELISA, Payment & Descoteaux, 1978). Horsfall & Curnen (1946) observed that there was a seasonal variation in the neutralizing activity of sera from cotton rats, and to a lesser extent, hamsters and mice, a much higher incidence of PVM antibody being detected in sera collected from January to June than in those collected from September to January. The animals tested at each time were new animals acquired from breeders so the antibody levels probably reflected the incidence of infection at different times of year. Horsfall & Curnen (1946) believed that the virus was present in mice in a latent form but Tennant et al. (1965) could find no evidence of latent infections and believed that the virus caused a low level of acute infections. The reason for the increased incidence of anti-PVM neutralizing activity observed when cotton rats or hamsters received intranasal inoculations of chick embryo cell suspensions or samples from patients with primary atypical pneumonia is unknown. The stimulation of antibody production by irrelevant antigens appeared to be dependent on a pre-existing antibody response in the animals but the authors did not study paired serum samples so it was not possible to tell if this was definitely the case.

Approximately one third of human sera were found to have neutralizing activity against PVM (Horsfall & Hahn, 1940). The antibody response to PVM in man has been studied in more detail by Pringle & Eglin (1986). More than 75 per cent of adult sera contained PVM neutralizing activity at a titre of greater than 40. The proportion of sera with similar levels of PVM neutralizing activity was found to increase with the age of children from which the samples were taken. Seroconversion (defined as >fourfold increase in titre) was observed in 4 of 108 paired serum samples from patients with undiagnosed respiratory infections. Sera from patients with Paget's disease of bone tended to have higher titres of PVM-neutralizing antibody than sera from patients with other bone diseases. This is similar to the results of immunofluorescence studies showing an increase in the frequency of antigens of other paramyxoviruses (e.g. measles virus, simian virus 5, parainfluenza 3, Baslé et al. 1985 and RS virus, Mills et al. 1981) in the osteoclasts of patients with this disease.

1.3.2 Experiments with PVM grown *in vivo*

Early studies on PVM used lung suspensions from infected mice as the source of virus. A large part of this early work concentrated on the haemagglutinating activity of the virus which was reviewed by Ginsberg (1951). PVM agglutinates murine and hamster erythrocytes (Mill & Dochez, 1944, 1945). The fluid expressed from lungs showed haemagglutination whereas lung suspensions from mice infected with PVM did not show

haemagglutinating activity unless the haemagglutinin was first dissociated from particulate material. Dissociation could be carried out with heat, high pH (Curnen & Horsfall, 1946, 1947) or low salt conditions (Davenport & Horsfall, 1950). The haemagglutinating activity was found to pass through bacteriological filters whereas the tissue associated form would not (Curnen & Horsfall, 1947; Curnen *et al.*, 1947). The haemagglutinin-lung tissue combination was reversible when the ionic strength was altered and in contrast to orthomyxoviruses and paramyxoviruses the haemagglutinin would recombine with the same cells (Curnen & Horsfall, 1946; Volkert & Horsfall, 1947). The haemagglutinin was later found to combine with mammalian tissues in which it had not been observed to multiply (Davenport & Horsfall, 1950) but not with chick embryo cells although the virus does multiply in these cells.

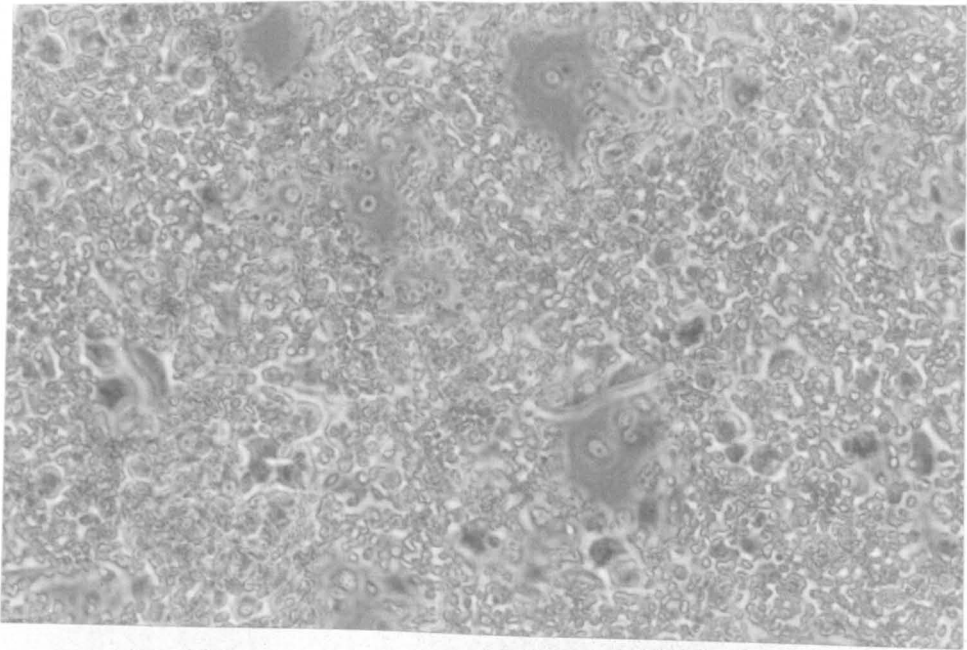
Ginsberg & Horsfall (1951a) and Horsfall & Ginsberg (1951) studied the multiplication of PVM in mouse lungs. Ninety per cent of the virus inoculated was not recoverable all of the infectivity having been lost within 12 hours of infection although haemagglutination activity could be demonstrated. Discrete cycles of replication occurred with the virus yield increasing by about 16 fold after each cycle. The rate of development of pneumonia was slower than the rate of multiplication of the virus and both these measurements reached limiting values. Multiplication of PVM and development of pneumonia are inhibited by some bacterial polysaccharides (Horsfall & McCarty, 1947; Ginsberg &

Horsfall, 1951b; Nemes & Hilleman, 1962) and by prior infection with a large dose of mumps virus (Ginsberg & Horsfall, 1952). These effects are considered to be due to the induction of interferon, polyI:C and double stranded RNA having similar effects (Nemes et al., 1969a&b; Tytell et al., 1970). PVM was more susceptible to these agents than other viruses tested including rabies, influenza A and B, Rous sarcoma virus, and Marek's disease virus.

1.3.3 Studies on PVM carried out in tissue culture

The first tissue culture system in which PVM was grown was minced chicken embryo cultures (Horsfall & Hahn, 1940). Tennant & Ward (1962) showed that PVM would grow in suckling hamster kidney cells and to a lesser extent in BS-C-1 cells but not in a variety of cells from man or mice. The first cell culture system used for more detailed studies of PVM was baby hamster kidney (BHK) cells (Harter & Choppin, 1967; Compans et al., 1967). This cell system was also used by Jakubik & Skoda (1974) who found that fibroblastic rather than epithelial cells gave the best yields of virus and that the best yields were obtained if the cultures were incubated at 32°C rather than 37°C and high multiplicities of infection of virus were used. In contrast Harter & Choppin (1967) found that PVM infected BHK-21 cells gave higher virus yields when incubated at 37°C than when incubated at lower temperatures. Cash et al. (1977) also used BHK cells when radiolabelling PVM. Cash et al. (1979a) found that satisfactory labelling of PVM polypeptides at 31°C was

a



b

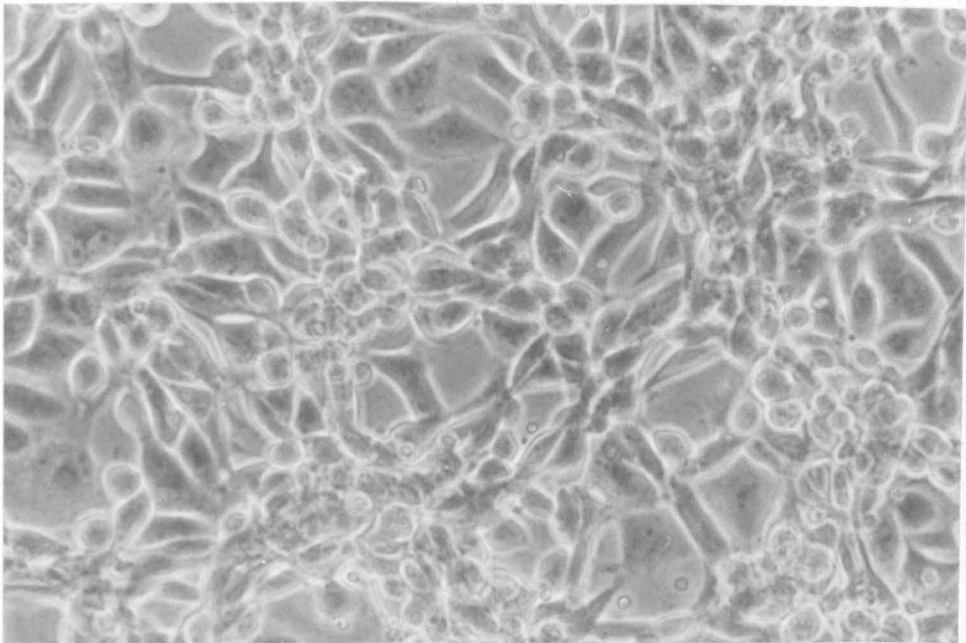


Figure 2 Haemadsorption with PVM infected BS-C-1 cells

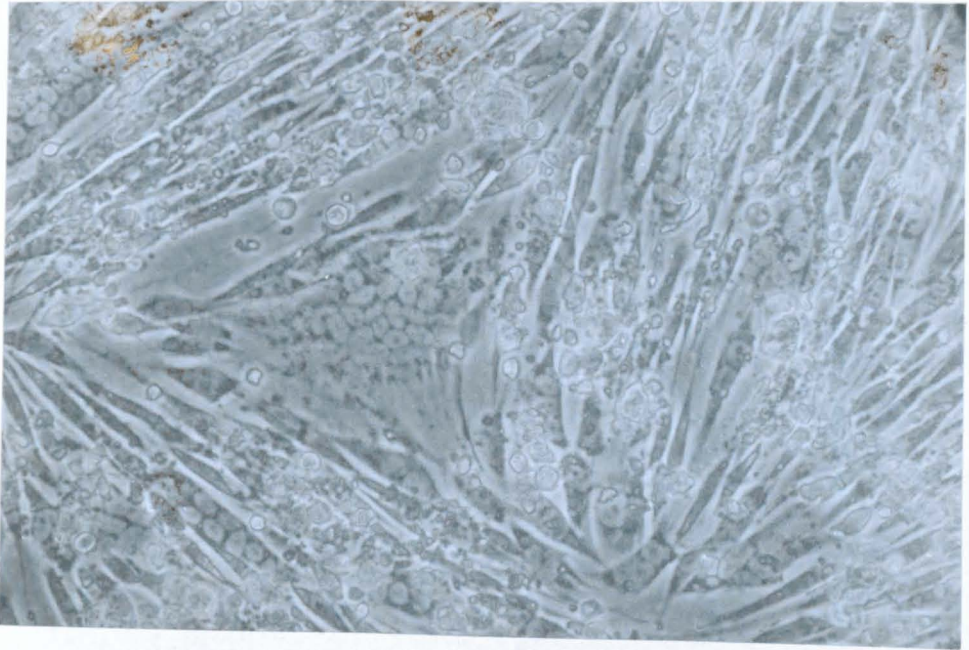
BS-C-1 cells infected 48 hours previously with PVM (m.o.i. of 4, a) or mock infected (b) were washed with PBS and exposed to murine erythrocytes in PBS (2 per cent v/v). The erythrocytes were washed off and the monolayers photographed revealing erythrocytes adsorbed to infected but not uninfected cells.

obtained using BS-C-1 cells in which more virus specific polypeptides could be identified. Haemadsorption has been demonstrated using murine erythrocytes and PVM infected BHK-21 cells (Compans *et al.*, 1967) or BS-C-1 cells (Figure 2). Cash *et al.* (1979a) found that like RS virus but unlike Sendai virus and measles virus PVM multiplied in enucleated cells (Follett *et al.*, 1975, 1976). Syncytium formation does not normally occur but has been observed using a high multiplicity of infection in BHK-21 cells (Figure 3).

The first assays of the virus were carried out using the dilution at which the virus caused CPE in 50 per cent of infected cultures as the titre (TCID₅₀ assay). Shimonaski & Came (1970) were able to perform a plaque assay using BHK cells with chymotrypsin or pancreatin but Jakubik & Skoda (1974) were not able to get reproducible results with this system. Cash *et al.* (1979a) assayed the virus on BS-C-1 cells where foci of darkly staining cells similar to but smaller than those produced by RS virus in BS-C-1 cells were observed.

Harter & Choppin (1967) found that haemagglutinating activity was more stable than infectivity. The replication of the virus was found to be insensitive to inhibitors of replication of DNA viruses and it was concluded that PVM was an RNA virus. Eosinophilic inclusion could be detected in the cytoplasm of infected BHK cells (Harter & Choppin, 1967). These inclusions were stained red by acridine orange indicative of the presence of single stranded RNA or DNA. Viral antigen was found to be localized in the cytoplasm. In

a



b

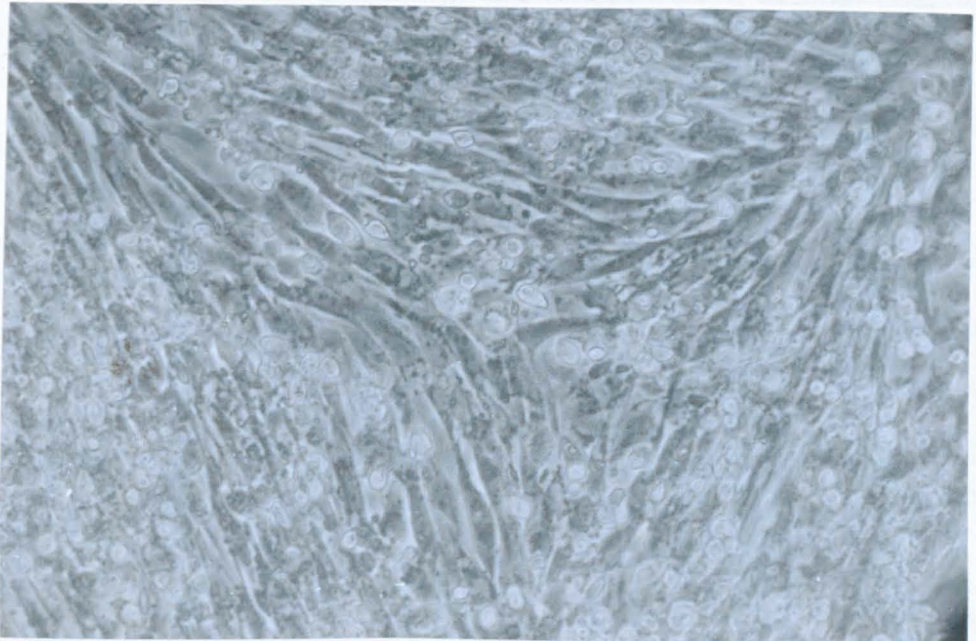


Figure 3 Syncytium formation in PVM infected BHK-21 cells

The figure shows PVM infected (at an m.o.i. of 10, a) and mock infected (b) BHK-21 cells at 3 days post infection. Syncytia are apparent in the infected but not the mock infected cells.

contrast to the virus grown in mouse lungs heating was not required to give the maximum haemagglutination titre. In contrast to influenza virus PVM was found to adsorb to erythrocytes that had been treated with neuraminidase. Pelleting of the virus and ammonium sulphate precipitation resulted in large reductions in the amount of infectious virus but an increase in the amount of haemagglutinating activity. Two peaks of infectivity were observed on potassium tartrate gradients, a visible band being associated with the minor but not the major peak. The haemagglutinating activity was not associated with the infectivity peaks suggesting that most of the haemagglutinating activity was associated with non-infectious virus (Harter & Choppin, 1967).

The structure and morphogenesis of PVM grown in Vero and BHK21 cells has been described (Berthiaume *et al.*, 1974; Compans *et al.*, 1967). PVM existed mainly as filamentous form 100nm in diameter and up to 3µm in length. A bulbous tip was sometimes observed at one end and spherical or pleomorphic particles have also been observed. The morphology is considered to be generally similar to that of RS virus but there are differences in that the surface projections of PVM are narrower and closer together than those of RS virus. Compans *et al.* (1967) found that the internal nucleocapsid structure was coiled inside filamentous forms of virus budding from the cell surface, whereas Berthiaume *et al.* (1974) found that the internal structures ran the length of the filaments. The diameter and

pitch of the helix in the internal components of PVM and RS virus were similar and different from the dimensions of paramyxovirus nucleocapsids (Table 1). Infected cells contained dense inclusions consisting of material with a thread-like appearance that were occasionally observed extracellularly probably due to disruption of infected cells. The dense material appeared to enter budding virions. Vesicles have also been observed on the surface of infected cells. PVM virions have been observed adsorbed to murine erythrocytes in electron micrographs and erythrocytes have been seen adsorbed to the surface of PVM infected cells but virus specific changes in cell morphology were not always evident at the points of contact.

1.3.4 Polypeptides of PVM

The polypeptides of PVM synthesized in infected BHK21 and BS-C-1 cells have been described (Cash et al., 1977 and 1979a). Two glycosylated and 10 unglycosylated polypeptides were observed in total using samples of radiolabelled BS-C-1 cell lysates and polyethylene glycol (PEG) precipitates of the supernatants of such cells. These authors identified one polypeptide as the nucleocapsid polypeptide and observed that 5 of the non-glycosylated polypeptides had similar mobilities to RS virus polypeptides. The polypeptides of PVM are considered in Sections 3 and 4 and will not be described in detail here.

Table 1 Structural features of paramyxoviruses¹

Property	RS virus	PVM	TRT virus	NDV
Diameter of spherical forms/nm	100-350	80-200	80-200	100-400
Length of filamentous forms/nm	<5000	<3000	1000-2000+	-
Diameter of filamentous forms/nm	60-110	100	-	-
Length of projections/nm	12-15	8-12	13-14	12
Spacing of projections/nm	10	6	-	-
Nucleocapsid diameter/nm	13.5-14	12-15	14	18
Nucleocapsid pitch/nm	6.5-7.0	6.5	7.0	5.5

1) Dimensions of the viruses of the genus *Pneumovirus* are compared with those of NDV, a representative of the genus *Paramyxovirus*.

References- Berthiaume et al. (1974); Collins & Gough (1988); Compans et al. (1967).

1.4 Turkey rhinotracheitis virus

Turkey rhinotracheitis is a disease of the upper respiratory tract of turkeys that has been described in many countries and reviewed by Lister & Alexander (1986). Morbidity is high with a mortality rate varying between less than 1 and 30 per cent (McDougall & Cook 1986). In the USA and West Germany the disease was considered to be caused by the bacterium *Alcaligenes faecalis*. However in other countries it has not been possible to associate this agent with the disease. A variety of viruses has been isolated from infected turkeys (Alexander et al., 1986) including adenoviruses, enterovirus-like viruses, reoviruses, rotaviruses and several unidentified viruses. Enveloped viruses have been isolated in avian tracheal organ cultures in several countries including the United Kingdom (McDougall & Cook, 1986; Jones et al., 1986; Wyeth et al., 1986), France (Giraud et al., 1986) and earlier in South Africa (Buys & du Preez, 1980 in Collins & Gough 1988). The isolates obtained by Wilding et al. (1986) and Wyeth et al. (1986) were found to be antigenically similar (Baxter-Jones et al., 1986). Isolates of this virus appear to be the most likely agent causing the primary infection at least in the United Kingdom. The viral infection predisposes the birds to secondary infections resulting in the symptoms of turkey rhinotracheitis. This virus has now been called turkey rhinotracheitis virus (TRT virus). The morphology and polypeptides of partially purified virus have been described (Collins et al., 1986b; Collins & Gough, 1988). Further work

on the polypeptides of this virus has been described by Ling & Pringle (1988) and is described in this thesis.

Collins et al. (1986b) using virus concentrated from the medium of tracheal organ cultures believed that the virus they observed had a similar morphology to viruses of the family *Paramyxoviridae*. They observed ribbon like structures some of which appeared to consist of two strands coiled around each other (or one strand coiled on itself) with a total diameter of 20nm but were not sure if these structures were virus specific. Later Collins & Gough (1988) described several forms of virus particles: roughly spherical forms were usually 80-200nm in diameter but sometimes they were several times larger; filamentous forms that sometimes exceeded 2 μ m in length also occurred. These pleomorphic forms of virus are similar to those associated with pneumoviruses (Berthiaume et al., 1974). The dimensions of the surface projections and nucleocapsid structures were similar to those reported for pneumoviruses (Table 1). The flexible appearance of the nucleocapsid resembled the appearance of PVM nucleocapsids more closely than that of RS virus nucleocapsids but the distinct surface projections of TRT virus were more similar to those of RS virus than those of PVM.

Collins et al. (1986b) compared the polypeptides of partially purified TRT virus grown in tracheal organ cultures and those of viruses representative of the *Orthomyxoviridae*, *Paramyxoviridae* and the *Coronaviridae*. The polypeptides were analysed by SDS-PAGE followed by staining

with Coomassie blue. The polypeptide profile obtained more closely resembled that of bovine RS virus than that of the Ulster strain of NDV, the parakeet/Netherlands/449/75 strain of avian paramyxovirus 3, the M41 and T avian coronaviruses or an orthomyxovirus, parrot/NI. The M_r s of the polypeptides that they reported appeared to be rather high by comparison with those expected for the pneumovirus polypeptides shown and these sizes have been revised in a later paper by the same group (Collins & Gough, 1988). The later paper describes similar work but the virus was grown in chick embryo cell cultures and the glycoproteins identified by staining with Schiff's reagent are described. Seven major virus specific polypeptides were identified with calculated M_r s of 54K, 47K, 42K, 37K, 36K, 31K and 14K when Coomassie blue staining was used. A 200K polypeptide was tentatively identified as being virus specific whereas four polypeptides with low M_r s were probably host cell contaminants. The glycoproteins had M_r s of 84K and 54K. The 84K polypeptide was more strongly labelled with Schiff's reagent although it was scarcely visible using Coomassie blue staining. Two other areas of heterogeneous staining were observed but no indication of their approximate mobilities was given.

1.5 Aims of the project

The polypeptides of RS virus have been studied in some detail, particularly the glycoproteins which appear to be the major viral antigens responsible for inducing immune responses in animals. The polypeptides of PVM have not been

described in such detail and their similarity to those of RS virus is not known. The study of RS virus polypeptides has been aided by the use of monoclonal antibodies directed against the viral proteins. This approach was therefore adopted for the study of PVM proteins. In particular the glycoproteins of PVM were studied to determine the extent of variation in the nature of the oligosaccharide-protein bonds and the processing of pneumovirus glycoproteins.

TRT virus has been suggested to be a fourth virus of the genus *Pneumovirus* on the basis of similarities in morphology and polypeptide profile to RS virus and PVM (Collins et al., 1986b; Collins & Gough, 1988). The polypeptides of TRT virus were characterized to compare the polypeptides of this virus and those of PVM and RS virus.

MATERIALS
AND
METHODS

MATERIALS

Suppliers names and addresses are given in Appendix 2.

2.1 Cell Lines

Balb/c ₃ T ₁₂	Flow Laboratories Inc.
BS-C-1	Professor C.R.Pringle, Department of Biological Sciences, University of Warwick, Coventry, UK
BHK-21	Flow Laboratories Inc.
NS-1	Dr. E.A.Jones, Department of Biological Sciences, University of Warwick
X63-Ag8.653	Flow Laboratories Inc.

2.2 Virus strains

Pneumonia virus of mice, strain 15 from the American type culture collection, and respiratory syncytial virus (RSS-2 strain originally isolated in Newcastle upon Tyne in 1970) were obtained from Professor C.R.Pringle. TRT virus was obtained from Dr. R. Gough (Central Veterinary Laboratory, Weybridge) by Dr. A. Easton and plaque purified 6 times by Professor C.R. Pringle.

2.3 Tissue culture media

Glasgow modification of Eagles medium with non-essential amino acids (GMEM) or 10 per cent Tryptose phosphate broth (GMEMB) and buffered with sodium hydrogen carbonate were provided by the virus group media preparation

laboratory. Double strength GMEM without methionine, valine, leucine, glutamine or sodium hydrogencarbonate, sterile distilled water, PBS, neutral red, trypsin and versene were also provided by this laboratory.

Aminopterin (1000X)

Aminopterin	25mg
Sodium hydroxide (0.1M)	10ml

When the aminopterin had dissolved the solution was made up to 142ml with distilled water, filter sterilized and dispensed in 5ml aliquots at -20°C

Antibiotics (1000X)

Crystapen	10 ⁶ Units
Streptomycin sulphate	1g
Distilled water	10ml
Filter sterilized and stored at 4°C	

Fusion medium

RPMI1640	500ml
Foetal calf serum	50ml
Sodium hydrogen carbonate solution (5%w/v)	20ml
Antibiotics	1ml
Glutamine (200mM)	5ml
ESG hybridoma growth factor	12.5ml

GMEMx

GMEM	1litre
Foetal calf serum	x%w/v
Glutamine (200mM)	20ml
Antibiotics	1ml

GMEMBx

As for GMEM with GMEMB in place of GMEM.

HAT medium (2X)

Fusion medium	100ml
Aminopterin (1000X)	0.1ml
HT (100X)	1ml
OR HAT medium supplement (50X)	4ml

HT (100X)

Hypoxanthine	136.1mg
Distilled water	100ml

Heated at 70°C for 1 hour to dissolve hypoxanthine,
allowed to cool and then added,

Thymidine	37.8mg
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Filter sterilized and stored in 10ml aliquots at -20°C

Hybridoma medium

Fusion medium	500ml
HT (100X)	5ml

Hybridoma freezing medium

Serum free myeloma medium	62ml
Foetal calf serum	30ml
Dimethyl sulphoxide	8ml

Methionine free medium

2X GMEM without leucine, valine, methionine, sodium hydrogencarbonate	500ml
Leucine (200mM)	10ml
Valine (200mM)	10ml
Glutamine (200mM)	10ml
Sodium hydrogencarbonate (5%w/v)	20ml
Antibiotics	1ml
Distilled water	to 1 litre

Myeloma medium

RPMI1640	500ml
Sodium hydrogencarbonate (5%w/v)	20ml
Foetal calf serum	50ml
Glutamine (200mM)	20ml
Antibiotics	1ml

Neutral red containing overlay

Made up as described below for overlay medium but 10ml neutral red solution (0.1%w/v) was added to solution B before mixing

Overlay medium (for plaque assays)

A	Noble agar	0.1%w/v
B	2X GMEM	100ml
	Foetal calf serum	3ml
	Antibiotics	0.2ml

A heated to melt agar and put at 45°C after allowing to cool

B warmed to 45°C

Equal volumes of A and B were mixed before use

Polyethylene glycol for cell fusion

Polyethylene glycol 1500	5g
RPMI1640+sodiumhydrogencarbonate	5ml
pH adjusted to about 8 with sodium hydrogencarbonate (5%w/v)	

OR used Polyethylene glycol 1500 in HEPES from BCL

Trypsin/versene

Trypsin solution	5ml
Versene	20ml

2.4 Buffers and solutions

Acid (for washing coverslips)

Hydrochloric acid	15ml
Nitric acid	5ml

Acrylamide solution

Acrylamide	28.5g
N,N-methylenebisacrylamide	1.5g
Distilled water	to 100ml

Crystal violet

Crystal violet	7.5g
Ethanol	500ml
Diluted 1 in 20 in distilled water for use	

Development solution for Western blots

A	HRP colour development reagent	60mg
	Methanol	20ml
B	Tris buffered saline (TBS)	
	Tris-HCl (1M, pH7.4)	100ml
	Sodium chloride (5M)	3.3ml
	Distilled water	to 1 litre

Immediately before use 60µl hydrogen peroxide was added to 100ml TBS

Solutions A and B were mixed immediately prior to use

ELISA substrate

Orthophenylenediamine	10mg
Hydrogen peroxide (100 volumes)	4µl
Sodium citrate buffer (0.1M, pH4.5)	10ml

Gel fixing solution

Methanol	500ml
Acetic acid	70ml
Distilled water	to 1 litre

Glutaraldehyde (for fixing plaque assays)

Glutaraldehyde solution (50%)	4ml
PBS	200ml

Glycosidase buffer A

EDTA (100mM)	20ml
2-Mercaptoethanol	2ml
Nonidet P-40	0.5ml
SDS (10 per cent w/v)	1ml
Sodium dihydrogen orthophosphate	2.83g
Distilled water	to 100ml
pH adjusted to 8.5 with orthophosphoric acid	

Glycosidase buffer B

Sodium dihydrogen orthophosphate	0.283g
Distilled water	100ml
pH adjusted to 6.0 with orthophosphoric acid	

Glycosidase buffer C

Calcium nitrate	0.131g
di-sodium hydrogen orthophosphate	4.25g
Nonidet P-40	0.5ml
Distilled water	to 100 ml
pH adjusted to 6.0 with phosphoric acid	

Isotonic lysis buffer

Magnesium chloride (1M)	0.3ml
Nonidet P-40	1.3ml
Sodium chloride (5M)	6ml
Trizma base	0.222g
Distilled water	to 200ml
pH adjusted to 7.8 with hydrochloric acid	

Lysis buffer (Western blotting)

Tris-HCl (1M, pH7.4)	2ml
NP40	1ml
EDTA	7.4mg
Distilled water	to 200ml
PMSF (100mM in ethanol) was added at 1:100 just before use	

MNH buffer

Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	246.4g
Sodium chloride	8.77g
HEPES	11.92g
Distilled water	to 1 litre
The pH was adjusted to 7.5 with 30%w/v sodium hydroxide	

NEPHGE sample buffer

Ampholines (pH3.5-10)	5 μ l
Ampholines (pH5-7)	20 μ l
2-mercaptoethanol	25 μ l
Nonidet P-40 (20 per cent v/v)	50 μ l
Urea (12M)	400 μ l

Overlay buffer

Ampholines (pH3.5-10)	2.5 μ l
Ampholines (pH5-7)	10 μ l
Distilled water	112 μ l
Urea (12M)	375 μ l

Paraformaldehyde

PBS	200ml
Magnesium chloride(1M)	0.10ml
Calcium chloride (1M)	0.18ml
Paraformaldehyde	8g

The suspension was heated on a magnetic stirrer until most of the solid had dissolved, filtered and stored in 5ml aliquots at -20°C

Phenol/Chloroform

Phenol (distilled, melted at 60°C)	100ml
Chloroform	100ml

The mixture was equilibrated by shaking with several changes (equal in volume to the phenolic phase) of phenol equilibration buffer.

Phenol equilibration buffer

EDTA (100mM)	2ml
Sodium chloride (5M)	6ml
Trizma base	0.222g
Distilled water	to 200ml
pH adjusted to 7.8 with hydrochloric acid	

Phenol extraction buffer

EDTA (100mM)	2ml
Sodium chloride	14g
SDS (10 per cent w/v)	20ml
Trizma base	0.222g
Urea	112g
Distilled water	to 200ml
pH adjusted to 7.8 with hydrochloric acid	

Phosphate buffered saline (PBS)

Potassium chloride	0.2g
Sodium chloride	8.18g
Potassium dihydrogen orthophosphate	0.2g
di-Sodium hydrogen orthophosphate	1.15g
Distilled water	1 litre

Phosphate buffered saline + bovine serum albumin (PBSBx)

PBS as above + x%w/v bovine serum albumin

Phosphate buffered saline + phenylmethanesulphonylfluoride
(PBSP)

PBS as above containing 1%v/v
phenylmethanesulphonylfluoride solution (100mM) in
ethanol

Phosphate buffered saline + Tween 20 (PBST)

PBS as above containing 0.1%v/v Tween20

Polyethylene glycol (PEG) for precipitating virus

PEG6000	36g
Distilled water	to 100ml

Ponceau S

Ponceau S	1g
Acetic acid	2ml
Distilled water	200ml

Radioactive ink

Ink	1ml
Culture medium containing [35 S]-methionine	1 μ Ci

RIP buffer

Triton X-100	2ml
Sodium chloride	1.758g
Potassium chloride	8.95g
Magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)	0.02g
Tris-HCl (1M, pH7.4)	2ml
Distilled water	to 200ml
PMSF (100mM in ethanol) was added to give a final concentration of 1mM immediately before use	

RIP wash buffer

Lithium chloride	21g
Tris-HCl (1M, pH8.9)	100ml
Distilled water	to 1 litre
The pH was adjusted to 8.5 with concentrated hydrochloric acid	

Running buffer

Trizma	6g
Glycine	4g
Sodium dodecyl sulphate	1g
Distilled water	1 litre

Sample buffer

Glycerol	3ml
Tris-HCl (1M, pH6.7)	1.5ml
2-Mercaptoethanol	1.5ml
Bromophenol blue	1ml
Sodium dodecyl sulphate	0.6g
Distilled water	3ml

Stacking gel buffer

Tris HCl (1M, pH6.7)	37.5ml
Sodium dodecyl sulphate (10%w/v)	3ml
Distilled water	to 300ml

Thin layer chromatography buffer

Acetic acid	10ml
Butan-1-ol	65ml
Distilled water	40ml
Pyridine	50ml

Thin layer electrophoresis buffer

Acetic acid	450ml
Distilled water	2400ml
Formic acid	150ml

Thin layer electrophoresis tracking dye

Acid fuchsin	0.1g
Orange G	0.2g
Thin layer electrophoresis buffer	10ml

Transfer buffer for Western blotting

Trizma base	3.03g
Glycine	36g
Methanol	200ml
Distilled water	800ml

Tris buffers (1M)

Trizma base	121.1g
Distilled water	to 900ml

The pH was adjusted to the required value with concentrated hydrochloric acid and the volume made up to 1 litre with distilled water

2.5 Methods

2.5.1 Growth of BS-C-1, BHK-21 and Balb/c $_3T_{12}$ cells

BS-C-1 and BHK-21 cells were grown in 1.5 litre roller bottles using GMEM5 and GMEMB5 media respectively. Cells were passaged at 4 day intervals at a ratio of 1:4. The cells were rinsed twice with versene and then with trypsin/versene solution, a small amount of which was left in the bottles. The cells were incubated at 37°C until all the cells became detached and they were then resuspended in the appropriate medium. Balb/c $_3T_{12}$ cells were passaged in a similar manner in 1 litre flat bottles.

2.5.2 Growth of virus stocks

Cloned virus (5×10^5 - 4×10^6 /ml) was used to infect confluent monolayers of BS-C-1 cells in 25cm^2 flasks (0.4ml/flask) or 1.5 litre roller bottles (1-2ml/bottle). The cells were incubated for 2 hours at 33°C and GMEM1 added, (5ml/ 25cm^2 flask or 15ml per roller bottle). The infected cells were incubated until CPE was extensive (3-4 days for RSS-2, 4-6 days for PVM), and then the cells were scraped into suspension and the suspension stored in 5ml aliquots at -70°C .

2.5.3 Plaque assay and plaque purification of the virus

Serial dilutions of virus were made in GMEM1 (10 fold for assays and 2 fold for plaque purification) and samples (0.1ml) added in duplicate to monolayers of BS-C-1 cells in 12 well (for assays) or 6 well (for plaque purification) tissue culture clusters. The cells were incubated for 1 hour at 37°C and overlay medium added (3ml/well or 7ml/well for 12 and 6 well clusters respectively). When this had set the plates were incubated at 37°C in a carbon dioxide gassed incubator for 5-6 days (for RSS-2) or 7-8 days (for PVM). The cells were fixed for 4 hours for assays with glutaraldehyde, the agar was then removed and the cells stained with crystal violet for 2-3 minutes. Excess stain was then washed off and foci of darkly stained cells counted with a low power microscope. When virus was to be isolated from widely separated foci a neutral red containing overlay was added instead of fixing the cells and the cells

incubated overnight in the dark. Foci were removed using a pasteur pipette inserted through the agar. A white background aided the visualisation of foci. The material in the pipette was resuspended in GMEM1 (0.1ml) and added to monolayers of BS-C-1 cells in 25cm² flasks. The monolayers were incubated at 33°C for 2 hours and GMEM1 then added (5ml/flask). The virus was harvested as described above up to 15 days after infection.

2.5.4 Neutralization assay

Serial 2 fold dilutions of serum or ascites were made and an equal volume of diluted virus added to each giving 100 infectious units in 0.2ml of the mixture. The mixtures were incubated overnight at 4°C and then added to monolayers. The assay was completed as described for the plaque assay. The numbers of foci obtained were plotted against the serum dilutions and the dilution giving 50% reduction of the number of foci defined as the neutralization titre.

2.5.5 Haemagglutination (HA) assay and haemagglutination inhibition (HI) assay.

Serial 2 fold dilutions of virus samples were made in PBS in 96 round bottomed well plates. Mice were killed by cervical dislocation and blood removed from the thoracic cavity after cutting the vena cavae. The blood cells were washed with PBS and the cells resuspended to give 5×10^8 cells per 10μl. 10μl of this suspension was added to each well and

the cells suspended by tapping the sides of the plates. The plates were left at room temperature for 2 hours and the HA titre determined as the highest virus dilution at which the cells did not form a pellet.

In the HI assay virus was diluted to an HA titre of 8 and equal volumes of this added to serum dilutions in 96 well plates. Murine erythrocytes were added as described for the HA assay and after 2 hours at room temperature the HI titre was determined as the highest serum dilution that inhibited haemagglutination.

2.5.6 Preparation of immunogen

- 1) Balb/c $3T_{12}$ cells were infected with PVM (5ml per 1 litre flat bottle at $>10^6$ infectious units per ml) and incubated at 31°C for 15 days after which time CPE became apparent. The cells were scraped into suspension and pelleted for 30 minutes at 4°C and 3000rpm using a 4x380 rotor of an MSE 6L centrifuge. The pellet was washed 3 times in PBS, resuspended at 2.5×10^7 cells per ml in PBS and stored at -70°C .
- 2) PVM was grown in 1.5 litre roller bottle cultures of BS-C-1 cells as described above. The cells were scraped into suspension and pelleted as described for Balb/c cells above. The supernatant was put on ice and made 6% with respect to polyethylene glycol 6000. The supernatant was left at 4°C overnight and the precipitate pelleted as described for the cells above. The surface of the pellet

was rinsed with GMEMO and it was then resuspended at 1 roller bottle of virus in 0.2ml of GMEMO. This was mixed with an equal volume of Freund's complete, (for the first injection) or incomplete (for subsequent injections), adjuvant for intraperitoneal inoculations.

- 3) PVM was prepared as a PEG precipitate as described above and then diluted in MNH to 30ml which was layered over 2ml sucrose in MNH (1.246gcm^{-3}) and 5ml sucrose in MNH (1.114gcm^{-3}) and centrifuged for 1 hour at 24000rpm in a Beckman SW27 rotor. The lower interface, containing most of the viral infectivity and haemagglutinin, was homogenized (30 strokes of a 1ml Dounce homogenizer), cleared for 1 minute at 10,000g and used as antigen for intravenous inoculations.

2.5.7 Enzyme-linked immunosorbent assay

BS-C-1 cells in 60 well HLA plates (10 μ l at 5×10^5 cells per ml), were infected with 10 μ l per well PVM or mock infected with GMEM1. The plates were incubated for 4 days at 33°C, washed with PBS and fixed for 30 minutes with 0.25% glutaraldehyde in PBS. The plates were then washed twice with PBS and non-specific binding sites blocked by incubation for 30 minutes with PBSB1. The plates were washed in PBS and stored at -20°C in PBS. Before use the plates were allowed to thaw and the PBS flicked off. The antibody was added to infected and uninfected and uninfected cells (10 μ l per well serum or ascites diluted in PBSB0.1 or

undiluted hybridoma supernatant) and the plates incubated for 3 hours at 37°C. The wells were emptied and washed 3 times with PBST. Biotinylated rabbit anti-mouse immunoglobulin diluted 1:1000 in PBSB0.1 was added and the plates incubated for 2 hours at 37°C. The wells were emptied and washed as before. Streptavidin diluted 1:500 in PBST was added and the plates incubated for 20 minutes at 37°C. The plates were washed again and biotinylated horseradish peroxidase diluted 1:2000 in PBSB0.1 was added followed by a further 20 minute incubation at 37°C. The plates were then washed 6 times with PBST and ELISA substrate added. The assay was read by eye a positive result being indicated by more colour development in the wells with infected as opposed to uninfected cells.

2.5.8 Immunization of mice

When Balb/c cells infected with PVM were used, 2 or 3 intraperitoneal (i.p) injections of 10^7 cells in 0.4 ml GMEMO were given at 1-2 week intervals. When PEG precipitates were used, 3 injections of 0.4 ml of the antigen adjuvant mixtures were given at 1-2 week intervals, followed after 3 weeks by 2 intravenous injections of partially purified virus (50µl per mouse), 7 and 4 days prior to fusion of the spleen cells.

2.5.9 Fusion of spleen cells with NS-1 or Ag8.653 myeloma cells.

Spleen cells were removed from mice receiving a final

boost of antigen 4 days previously and rinsed in ethanol. The fusions were carried out essentially as described by Galfré *et al.* (1977). The spleen was placed in a Petri dish containing 10ml serum free myeloma medium, one end cut and the cells teased out with sterile curved forceps. The outer coat of the spleen was then discarded and the cells suspended in the medium. Large clumps were allowed to settle and the suspension added to 15 ml myeloma medium containing 10^7 myeloma cells. The cells were pelleted for 5 minutes at position 3 on the MSE minor bench centrifuge in conical bottomed bottles. The supernatant was completely removed and the cells resuspended over a 1 minute period in 1ml of polyethylene glycol made up as described above or obtained from BCL. Serum free medium (1ml) was then added at a similar rate followed by 10ml of this medium over a period of 5 minutes. A further 10ml of this medium was then added and the cells pelleted for 5 minutes as described above. The cells were resuspended in about 50ml of fusion medium and distributed into 96 well tissue culture plates at 0.1ml per well. The cells were incubated for 24 hours at 37°C and double strength HAT medium added (0.1ml per well). The cells were then left undisturbed at 37°C for a further 9 days. Supernatants of wells containing colonies were screened for anti-PVM antibody by ELISA 10-20 days after fusion.

2.5.10 Propagation, cloning and freezing of hybridoma cells

Cells from wells giving positive reactions in ELISA tests were transferred to 24 well clusters containing 10^6

normal mouse spleen cells per well. When the cells showed good growth the medium was removed and placed in 1.5ml polypropylene tubes at -20°C . The cells were resuspended in cold hybridoma freezing medium (0.1ml per well) and placed in 2ml cryogenic storage vials on ice. The vials were placed in a vacuum flask or thick walled polystyrene box at -70°C overnight and then placed in liquid nitrogen. The cells were thawed by adding 1.5ml warm hybridoma medium from 12 well clusters of spleen feeder cells (10^6 per well) and the medium replaced in the wells with the feeder cells.

The cells were cloned by limiting dilution. The cells were diluted to give expected cell numbers per well of 50, 5 and 1 cell per well for the first cloning and 10, 1 and 0.2 cells per well for subsequent cloning steps. The plates were incubated in humidified boxes at 37°C and the supernatants of wells containing single colonies screened for antibody production by ELISA 8-20 days later. Some of the cells were recloned immediately whilst the rest were grown up in 24 well clusters with spleen feeder cells. When large colonies were obtained in 24 well clusters the cells were transferred to 5ml hybridoma medium in 25cm^2 flasks. Cells growing in flasks were either frozen (0.5ml hybridoma freezing medium per flask) or, after the cells had been cloned 3 times, passaged at 2-3 day intervals (by dilution 1 in 10 in fresh hybridoma medium). Cells were grown up to 200ml for freezing (5 vials, 1ml per vial) or for inoculation into mice.

2.5.11 Production of ascites

Mice were inoculated i.p. with 0.4 ml per mouse 2,6,10,14-tetramethylpentadecane 1 to 4 weeks prior to inoculation with 10^7 hybridoma cells in serum free myeloma medium. Ascitic fluid was withdrawn 2 or 3 times before killing the mice by cervical dislocation. The ascites was clarified for 10 minutes at the maximum speed on the MSE minor bench centrifuge and stored at -20°C or -70°C .

2.5.12 Labelling of virus infected cells

BS-C-1 cells in 6 well tissue culture clusters (10^6 cells per well) were infected at a multiplicity of infection (m.o.i) of 1 to 4 and the inoculum replaced after incubating for 2 hours at 33°C by GMEM1, (3ml per well). The cells were then incubated at 33°C for 2 days and the medium replaced with 2ml per well methionine free medium. The cells were incubated for from 1 to 6 hours in this medium before replacing it with 0.5 ml per well of methionine free medium containing 50-1000 μCi per ml ^3S -methionine. When ^3H -glucosamine was used, serum free medium was used in place of methionine free medium and ^3H glucosamine used at 100 μCi per ml.

The cells were scraped into the medium after 18-24 hours and pelleted for 20 seconds at 10,000g. The supernatant was made 6 per cent with respect to polyethylene glycol 6000 and 1mM with respect to PMSF and put at 4°C for from 2 hours to overnight. The precipitate was collected by pelleting for 5 minutes at 10,000g. The cells were washed

twice in PBSP and lysed in RIP buffer for 30 minutes on ice. Debris was removed by centrifugation for 5 minutes at 10,000g and the supernatants transferred to new tubes.

2.5.13 Pulse chase experiments

BS-C-1 cells in a 6 well tissue culture clusters were infected as described above (2.5.12) for other labelling experiments. 2 or 3 days after infection when CPE was extensive the medium was removed and for experiments with [^{35}S]-methionine the cells were incubated with methionine free medium (3ml per well) for 6 hours prior to labelling. 1-[^3H]-glucosamine hydrochloride was diluted with an equal volume of double strength GMEM and this solution diluted with GMEM0 to give a radioactive concentration of 500 μCi per ml. L-[^{35}S]-methionine was diluted in methionine free medium to give a radioactive concentration of 500 μCi per ml. Pulse labelling was performed for 10 or 20 minutes at 33°C using 0.2 ml label solution per well. Pulse samples were harvested as described above for other samples except that the cells were only washed once in PBSP. Cells that were to be left for a chase period were washed 3 times with GMEM5 after labelling and then incubated at 33°C for the indicated chase periods with GMEM5 (0.5ml per well). When [^{35}S]-methionine was used as the label, 200 times the usual concentration of methionine was used in the medium used for washing the cells. The samples were harvested in the same way as the pulse samples.

2.5.14 Preparation of Immuno-Precipitin for immunoprecipitation.

Immuno-Precipitin (formalin fixed *Staphylococcus aureus*, BRL) was pelleted 10 minutes at 4000g at 4°C. the bacteria were resuspended in PBS containing 10 per cent 2-mercaptoethanol and 3 per cent SDS and incubated in a boiling water bath for 30 minutes. This procedure was repeated and the bacteria resuspended in RIP buffer and stored at 4°C. This procedure was recommended by the manufacturer to reduce non-specific binding.

2.5.15 Radioimmunoprecipitation

PEG precipitate (1 well equivalent resuspended in 100µl RIP buffer) and cell lysates were put on ice for 30 minutes with 50µl Immuno-Precipitin and 10µl of ascites or serum not containing anti-viral antibody. The bacteria were pelleted for 5 minutes at 10,000g. Ascites or serum (3-30µl was added to each supernatant and put at 4°C for from 3 hours to overnight. Immuno-Precipitin in RIP buffer was added to each tube (50-300µl) and pelleted at 10,000g for 1 minute after incubation for 30 minutes on ice. The bacteria were washed 3 times in immunoprecipitation washing buffer using 1ml volumes and pelleting for 30 seconds at 10,000g, before resuspending in 40µl boiling buffer and either storing at -20°C or boiling for 3 minutes and loading onto gels.

**Table 2 Composition of polyacrylamide gel solutions
(gradient gels)**

Reagent	Amount used in		
	6% gel	15% gel	Stacking gel
Acrylamide-Bis	4.8ml	12 ml	5ml
Tris-HCl(1M, pH8.9)	9.0ml	9.0ml	
Tris-HCl(1M, pH6.7)			3.75ml
Distilled water	9.81ml	0.36ml	20.7ml
Glycerol		2.4ml	
SDS (10%)	0.24ml	0.24ml	0.3ml
Ammonium persulphate (10%w/v)	0.15ml	0.075ml	0.25ml
TEMED	0.01ml	0.01ml	0.012ml

2.5.16 Polyacrylamide gel electrophoresis (SDS-PAGE)

The discontinuous buffer system of Laemmli (1970) was used. 6-15 per cent gradient resolving gels were used as described by Marsden et al. (1976). The composition of the stacking gel and resolving gel solutions are indicated in Table 2. In some experiments 15 per cent single concentration gels were used, the composition of the gel solutions used for these gels are shown in Table 3. The gels were electrophoresed using 250x200mm plates with 1.5 mm spacers in a vertical slab gel apparatus at a constant current of 12mA per gel until the dye front reached the bottom of the gel.

2.5.17 Two dimensional NEPHGE/SDS-PAGE

BS-C-1 cells were grown in 96 well tissue culture plates (2.5×10^5 cells per ml, 0.1 ml per well) and infected 24 hours later with PVM, RS virus or TRT virus (200 μ l per well at titres of 4×10^6 , 8×10^6 and 2×10^6 per ml respectively). After a 2 hour adsorption period the cells were incubated at 33°C for 48 hours (for PVM and RS virus infected cells), or 72 hours (for TRT virus infected and uninfected cells) with GMEM1. The cells were incubated in methionine free medium containing actinomycin D (2.5 μ g per ml) for six hours before labelling with [35 S]-methionine (1mCi per ml, 50 μ l per well) overnight. The supernatants were removed clarified for 1 minute at 10,000g and precipitated overnight at 4°C with 6 per cent (final concentration) PEG6000. The precipitate was resuspended in

Table 3 Composition of polyacrylamide gel solutions
(15 per cent gel)

Reagent	Volume used for:-	
	Stacking gel	Resolving gel
Acrylamide-Bis (2)	2.5ml	35ml
Tris-HCl (1M, pH8.9)		28ml
Tris-HCl (1M, pH6.7)	3.75ml	
Distilled water	8.45ml	5.6ml
SDS (10 per cent w/v)	0.15ml	0.7ml
Ammonium persulphate	0.15ml	0.7ml
(10 per cent w/v)		
TEMED	0.006ml	0.02ml

50µl NEPHGE sample buffer. The cells were solubilized in NEPHGE sample buffer. Two dimensional NEPHGE/SDS-PAGE was carried out as described by O'Farrell et al. (1977). The samples were clarified at 10,000g for 5 minutes before loading onto the first dimension NEPHGE gels. The composition of the NEPHGE gel mixture is shown in Table 4. The samples were overlayed with NEPHGE overlay buffer (20µl per tube) and the tubes filled with phosphoric acid (0.01M). The gels were electrophoresed in a rod gel apparatus with phosphoric acid (0.01M) in the upper reservoir and sodium hydroxide (0.02M) in the lower reservoir. The gels were electrophoresed at 400V for 6 hours with the cathode at the bottom. The gels were extruded by forcing water between the gel and the tube with a syringe and blowing the gel out with a pipette filler. Before the gel had completely emerged from the tube the acid end was marked with a piece of fine wire.

The second dimension gel was a 6-15 per cent gradient gel with a 5 per cent stacking gel. A comb was not used in the stacking gel a small piece of spacer being used to form a well for marker polypeptides and the gel overlayed with water. The gel surface was washed with melted agarose (1 per cent w/v in stacking gel buffer) and the first dimension gel sealed in with the agarose. The gels were electrophoresed at 12 mA overnight and processed for autoradiography (cell lysate samples) or fluorography (all other samples).

2.5.18 Western blotting - preparation of samples

1.5 litre roller bottles of BS-C-1 cells were infected

**Table 4 Composition of polyacrylamide gel solutions
(NEPHGE gels)**

Reagent	Quantity
Acrylamide-Bis	1.33ml
Urea	5g
Distilled water	2.95ml
Ampholines (pH3.5-10)	0.5ml
Nonidet-P40	1ml
(20 per cent v/v)	
Ammonium persulphate	0.02ml
(10 per cent w/v)	
TEMED	0.014ml

at an m.o.i. of 0.1 to 0.25 and the virus allowed to adsorb for 2 hours at 37°C. GMEM1 (15ml per bottle) was added and the incubation continued until CPE was extensive. The cells were then scraped into suspension and pelleted at 4000g at 4°C for 10 minutes. The supernatant was made 6 per cent w/v with respect to PEG 6000 and 1mM with respect to PMSF and incubated for 2 hours at 4°C before pelleting for 30 minutes at 4000g and 4°C. The precipitate was suspended in PBSP at 1 roller bottle per 0.2 ml and stored at -20°C. The cells were washed twice with PBSP and lysed in lysis buffer at 1 roller bottle per ml for 10 minutes on ice before storage at -20°C. Samples (10µl per well) were diluted to 40µl per well with boiling buffer.

2.5.19 Western blotting- transfer of protein to nitrocellulose, staining and immunological detection of antigen

Gels were equilibrated with transfer buffer for 30 minutes and then placed on paper (Whatman 3MM) on a fibre pad resting on half of a gel holder of the trans-blot™ apparatus (Bio-rad). The assembly was completed with nitrocellulose, paper and another fibre pad (all soaked in transfer buffer) according to the manufacturers instructions. The gel holder was inserted in the tank containing transfer buffer with the nitrocellulose towards the anode. Transfer was performed for 3 hours at a constant voltage of 70V. The gel assemblies were removed from the tank, opened, and the nitrocellulose sheets stained for 5 minutes with Ponceau S and destained for 3 minutes with

distilled water. The positions of marker bands was indicated using a pencil and the sheet cut into strips. The nitrocellulose was then destained by four 10 minute washes in distilled water, any residual stain being removed during the blocking step. Blocking of non-specific binding was carried out using PBSB3 overnight at 4°C. Blots were washed (5 times for 5 minutes each for this and all subsequent steps unless otherwise indicated) and antibody added (1 in 250 dilution in PBSB1 of serum or ascites or undiluted tissue culture supernatant). The blots were incubate at room temperature for from 1 to 3 hours and then washed. Biotinylated second antibody (directed against the first antibody) diluted 1 in 500 in PBSB1 was incubated with the blot for 1-3 hours and the blot washed. Biotinylated horseradish peroxidase-streptavidin complex (1 in 400 in PBSB1) was then incubated with the blot for 30 minutes at room temperature. The blot was then washed 3 times for 5 minutes each with PBST and twice briefly with PBS before adding the development solution. When bands became apparent the blot was quickly washed with water and allowed to dry.

2.5.20 Experiments with tunicamycin and monensin

In the experiments with tunicamycin or monensin these inhibitors were added to all media at concentrations of 2.5µg per ml and 0.8µM respectively or as indicated.

2.5.21 Immunofluorescence

Glass coverslips were washed once with acid, 3 times

with ethanol and then stored in ethanol. Coverslips were flamed and placed in 6 well clusters (2 coverslips per well). Alternatively 12 well tissue culture slides (Flow Laboratories Inc.), were washed once with detergent followed by rinsing in distilled water and sterilization by heat treatment (160°C for 4 hours). BS-C-1 cells were grown overnight on the coverslips or slides, (4×10^5 cells per well for the coverslips or 5×10^5 cells in a 9cm diameter Petri dish for the slides). The cells were infected at a m.o.i. of 0.2 or mock infected with an equal volume of GMEM1 and 24 hours later washed 3 times with PBS at 37°C followed either by fixation with cold (-20°C) acetone for 10 minutes or incubation on ice for 30 minutes with antibody. The antibodies were used as 1 in 100 dilutions of ascites or serum in PBSB0.1 or undiluted tissue culture supernatant. Fixed cells were allowed to dry and stored dry at -20°C . Antibody was added to fixed cells at 37°C for 1 hour. The cells were then washed 3 times in cold PBS for unfixed cells or PBS at ambient temperature for fixed cells and fluorescein isothiocyanate conjugated rabbit anti-mouse immunoglobulin antibody diluted 1 in 60 in PBSB0.1 added. The incubation was carried out for 30 minutes on ice for unfixed cells and for 1 hour at 37°C for fixed cells. The cells were then washed 3 times in PBS (at 4°C when unfixed cells were used). Fixed cells were mounted using 80 per cent glycerol and 20 per cent saline and viewed with a Nikon Optiphot microscope with an incident ultraviolet light source at 1250X magnification. Photography was carried out

using exposures of 60-200 seconds with Ektachrome 160 colour reversal film. Unfixed cells were fixed for 15 minutes with paraformaldehyde, washed in PBS, mounted and examined as described above.

2.5.22 Partial proteolysis of viral proteins

Two methods were used that gave similar results, the first method (from McCrae & Joklik, 1978) possibly giving less streaking on the gels. Bands were excised from dried gels using an autoradiogram as a template and placed in 0.5ml polypropylene tubes. The gel slices were ground up in stacking gel buffer (140 μ l per tube) and left to swell for 10 minutes. Undigested samples were then placed on ice. 10 μ l of protease at either 1.5mg per ml or 150 μ g per ml was added to each tube containing samples to be digested and the tubes incubated at 37°C for 30 minutes. The tube tops were then punctured with a large bore needle and 2 holes made near the bottoms of the tubes with 25g needles after cleaning them with industrial methylated spirit. The tubes were placed in 1.5 ml polypropylene tubes without lids and centrifuged at 2000g at 4°C for 5 minutes. Boiling buffer was added to the liquid collected in the larger tubes and the samples transferred to new tubes before boiling for 3 minutes and loading onto gels.

An alternative procedure was that of Cleveland et al. (1977). The gel slices were rehydrated in stacking gel buffer and placed in wells of a second gel. The slices were overlayed with 20 μ l per slot of 20 per cent v/v glycerol in

stacking gel buffer. This was then overlaid with 10 μ l of stacking gel buffer containing an appropriate protease concentration, 10 per cent glycerol and 0.15mg bromophenol blue per ml. The gel was electrophoresed at 40 mA until the dye was about two thirds of the way down a 5 cm stacking gel and the power was then turned off for 40 minutes to allow digestion to occur. The gel was then electrophoresed overnight at 12 mA.

2.5.23 Fluorography and autoradiography

Gels were fixed with 2 changes of fixer the gels being left in the second change overnight. Fluorography was carried out using AmplifyTM for 30 minutes. Gels were dried under vacuum for 1.5 hours using a Bio-rad gel drier. Gels were exposed to pre-flashed X-ray film at -70°C. When autoradiography was used to detect bands for partial proteolysis the gels were dried down immediately after electrophoresis and the film was not pre-flashed. Calculation of M_r s was carried out by comparison of the mobilities of the polypeptides with those of radiolabelled molecular weight markers. A sonic digitizer was used to record the mobilities and the M_r s of the polypeptides were calculated using a third degree log curve using the Beckman Microgenie Software.

2.5.24 Two dimensional tryptic peptide maps of PVM polypeptides

PVM infected BS-C-1 cells were labelled from 48 hours to 72 hours after infection in the presence or absence of

tunicamycin (2.5 μ g per ml). The radiolabels used were [35 S]-methionine (500 μ Ci per ml) and [4,5- 3 H]-leucine (750 μ Ci per ml). The polypeptides were immunoprecipitated with murine anti-PVM serum and separated by SDS-PAGE using a 6-15 per cent resolving gel as described in Sections 2.5.15 and 2.5.16. Radioactive ink spots were used to mark the dried gel so that the fluorograph could be aligned with the gel. Tryptic peptide mapping was carried out as described by Elder et al. (1977). The bands to be digested were cut out of the gel using the fluorograph as a template and washed 4 times for 30 minutes with 25 per cent propan-2-ol and left overnight in 10 per cent methanol. The gel slices were lyophilized and incubated for 24 hours at 37°C in ammonium hydrogen carbonate (0.05M) containing trypsin (50 μ g per ml). The liquid was removed from the tubes, lyophilized and reconstituted in 50 μ l per sample of thin layer electrophoresis buffer. Samples and TLC tracking dye (4 μ l of each) were loaded 40mm from opposite ends and 40mm from one edge of a 200X200mm cellulose thin layer chromatography plate. The plate was placed on a glass plate on a horizontal gel tank. The chambers of the tank were filled to just touch the glass plate with thin layer electrophoresis buffer and the plate was sprayed with this buffer. A strip of dialysis membrane soaked in the same buffer was placed along the two ends of the plate and wicks of 3 thicknesses of 3MM paper were placed on top of the membrane and allowed to dip into the buffer in the reservoirs. The tanks were covered with cling film and the samples run at 1000V at 4°C until the

leading dye reached the point of application of the sample. The sample migrated towards the cathode. The plates were allowed to dry at room temperature overnight. The second dimension was carried out using thin layer chromatography the edge of the plate parallel to the direction of the first dimension and nearest to the spots was placed in TLC buffer in a glass tank lined with 3MM paper soaked in this buffer. When the leading dye was about 10mm from the end of the plate the chromatographs were removed and allowed to dry at room temperature (in a fume hood) overnight. The chromatographs were sprayed with diphenyloxazole (PPO, 22 per cent w/v in diethyl ether) in a fume hood. The coating was checked by visualization under an ultraviolet lamp. The plates were then exposed to pre-flashed X-ray film at -70°C .

2.5.25 Digestion of glycoproteins with glycopeptidase F and endo- α -N-acetylgalactosaminidase.

PVM infected cells incubated in the presence or absence of tunicamycin (2.5 μg per ml) were labelled from 48 to 72 hours post infection. The labelling was carried out in the presence or absence of tunicamycin with either [^{35}S]-methionine or [^3H]-glucosamine each at 100 μCi per ml. RS virus infected cells were labelled with [^3H]-glucosamine in the absence of tunicamycin in a similar way. The samples were immunoprecipitated as described in Section 2.5.12 and 2.5.15 using 50 μl of monoclonal antibody 19/1/C9, 40 μl of murine anti-PVM or, for RS virus infected cells, 30 μl of bovine anti-RS virus serum with samples from 1×10^6 cells.

Table 5 Composition of glycosidase digestion mixtures

Reagent	Volume (μ l) used in each digestion				
	U ¹	N	M	O	N+O
Glycopeptidase F ²		20			4
Mixed enzymes ³			1	1	1
Neuraminidase ⁴			1	1	1
Endo- α -N-acetyl- galactosaminidase				1	1
Distilled water	8				
Buffer A ⁵	12				
Buffer B			18	17	
Buffer C					13

- 1) U- control, N- digestion to remove N-linked sugars, M- digestion with mixed exoglycosidases, O- digestion to remove O-linked sugars, N+O- digestion to remove N- and O- linked sugars. The volumes of solutions refer to 20 μ l reaction volumes, where total volumes of 10 μ l were used the volumes indicated were halved.
- 2) 100 μ l diluted to 233 μ l with glycosidase buffer A.
- 3) Mixed glycosidases from *Turbo cornutus* at 0.1 mg per ml.
- 4) Neuraminidase at 1 unit per ml.
- 5) The buffers are the glycosidase buffers A, B and C.

The polypeptides were separated by SDS-PAGE and the bands located and excised using fluorographs of the gels as templates. Four bands of each polypeptide were eluted electrophoretically through stacking gels in 10X0.5cm gel tubes with dialysis tubing at the bottom. The dialysis tubing was attached to the gel tubes with grommets and the other end tied off. The gel slices were rehydrated in sample buffer and overlayed in the gel tubes with 200 μ l of this buffer. The gels were run until the bromophenol blue entered the dialysis tubing. The dialysis membrane was tied off at the top and the samples dialysed against 5 changes of ammonium hydrogencarbonate (0.1M). The samples were divided into 5 aliquots and lyophilized.

Samples that were digested with glycopeptidase F were incubated for 17 hours at 37°C after resuspension in 20 μ l (for [³⁵S]-methionine labelled samples) or 10 μ l (for [³H]-glucosamine labelled samples) of glycopeptidase F (diluted to 60U per ml in glycosidase buffer A). Undigested samples were incubated under similar conditions with buffer A only (Table 5).

Digestion with Endo- α -N-acetylgalactosaminidase was performed in the presence of exoglycosidases since this enzyme only cleaves unsubstituted disaccharides from polypeptide chains. Therefore a sample digested with these enzymes alone had to be included as a control. The composition of the mixtures used is shown in Table 5. The incubations were carried out at 37°C overnight. Sample buffer (20 μ l) was added to all the digestions and the

samples heated to 100°C for 2 minutes prior to analysis by SDS-PAGE with 6-15 per cent gradient resolving gels and fluorography of the dried gels.

2.5.26 Preparation of cellular RNA

BS-C-1 cells were infected with PVM (m.o.i. of 1), RS virus (m.o.i of 2) or TRT virus (m.o.i of 0.5) or mock infected and the inoculum replaced with GMEM1 after 2 hours adsorption at 37°C. The cells were incubated at 37°C for 24 hours and the medium replaced with similar medium containing actinomycin D (2.5µg per ml). The cells were scraped into suspension and pelleted at 4000g for 5 minutes at 4°C, 48 (PVM and RS virus infected cells) or 72 hours (TRT virus and mock infected cells) after infection. The RNA extraction was carried out using a method based on that of Kumar & Lindberg (1972), (Dr. A. Easton, personal communication). All the glassware was treated at 200°C for 2 hours and the solutions were autoclaved. The cells were lysed by resuspension and incubation for 10 minutes on ice using isotonic lysis buffer. The cell debris was pelleted for 5 minutes at 4000g and 4°C. The cell lysate (5ml containing 4×10^6 cells) was added to a mixture of 5ml equilibrated phenol/chloroform and 5ml of phenol extraction buffer. The mixture was shaken and the upper (aqueous) phase removed and re-extracted twice with phenol/chloroform. The final aqueous phase was precipitated overnight with 2.5 volumes of ethanol at -20°C. The precipitate was resuspended in 100µl of DEPC treated water and diluted to 1mg per ml. The concentration of RNA

was calculated on the basis that an absorbance of 20 units at a wavelength of 260nm was equivalent to that due to an RNA solution at a concentration of 1mg per ml.

2.5.27 *in vitro* translation of cytoplasmic RNA

RNA extracted from infected or uninfected cells extracted as described in Section 2.5.26 was translated *in vitro* using commercially prepared rabbit reticulocyte lysate (Amersham International). [³⁵S]-methionine (250μCi) was freeze dried resuspended in the reticulocyte lysate (100μl) and divided into 8μl aliquots. RNA (1μl and DEPC-treated water (1μl) were added to each tube and the mixtures were incubated at 30°C for 90 minutes. For samples to be analysed directly 30μl of sample buffer or NEPHGE sample buffer were added to each tube. When samples were immunoprecipitated 50 μl of RIP buffer were added to each tube and immunoprecipitation performed as described in Section 2.5.15.

3 RESULTS

3.1 POLYPEPTIDES OF PVM

3.1.1 Introduction

The polypeptides of RS virus, the type member of the genus *Pneumovirus*, were described in Section 1. The description of PVM polypeptides have been limited to preliminary observations without the aid of specific antibodies to confirm their virus specificity.

The aims of the experiments described in this section were to establish common and specific features of the polypeptides of the pneumoviruses, to verify and extend the observations of Cash et al. (1979) on PVM polypeptides. RS virus was used as a control in most of the experiments since it is the established prototype of the genus. Polyethylene glycol precipitates of cell supernatants were used extensively in the work described in this and subsequent sections to identify proteins detected in clarified cell supernatants. This procedure described in Section 2.5.12 was a convenient way of concentrating polypeptides present in cell supernatants for analysis by SDS-PAGE either directly or following immunoprecipitation. These polypeptides could have represented virion polypeptides, secreted protein or protein released from disrupted cells. This approach was used since virus purification was difficult and unsatisfactory. The results presented in this section describe the properties of the PVM polypeptides in terms of estimated relative molecular masses and relative charges. Polyclonal antisera were used to establish the viral specificity of the polypeptides and to examine antigenic

cross reactions between PVM and RS virus.

3.1.2 Polypeptides of PVM synthesized *in vivo*

The polypeptides observed in [³⁵S]-methionine labelled PVM infected cell lysates and the polyethylene glycol precipitates of the supernatants of these cultures are shown in Figure 4. PVM infected BS-C-1 cells were labelled from 48 hours to 96 hours post infection in the presence of actinomycin D as described in the figure legend. Actinomycin D was used to inhibit cellular mRNA synthesis and hence reduce the incorporation of labelled amino acid into cellular protein. Figure 4b is an overexposure of the gel to reveal the weakly labelled polypeptides with high mobilities and Figure 4a is a shorter exposure of the same gel. The 39K, 25K, 23K, 20K, 19K, 18K, 16K, 12K and 9K polypeptides were designated according to their relative molecular masses (M_r , $\times 10^{-3}$ e.g. the 39K polypeptide had a calculated M_r of 39,000). The M_r s were obtained by comparing the mobilities of the polypeptides with those of protein molecular weight standards using a digitizer and Microgenie software (Section 2.5.23). The G1, G2 and F1 polypeptides are virus specific glycoproteins (see Section 3.3). The F1, N and M designations were given to PVM polypeptides which on completion of studies described in this thesis were considered to have similar properties to RS virus polypeptides with corresponding names. No non-structural polypeptides could be identified. The G1, G2, F1, 25K, 16K and 9K polypeptides were only identified in the PEG

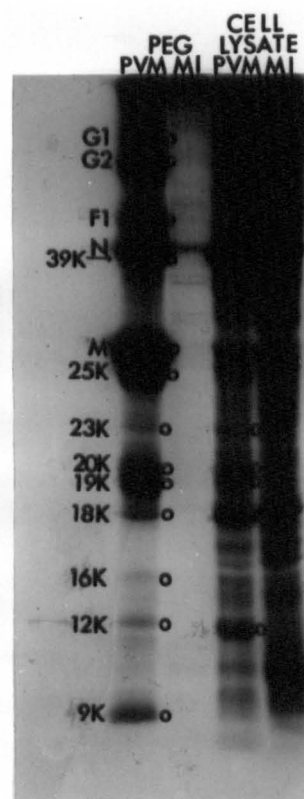
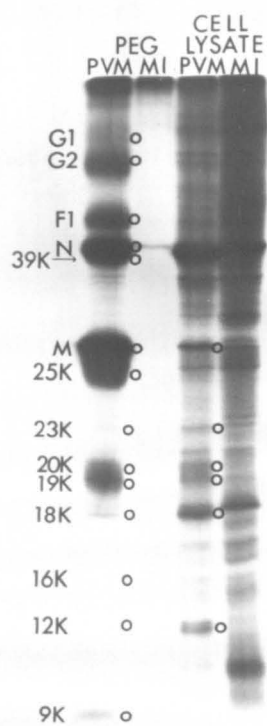
Figure 4. Polypeptides of PVM synthesized *in vivo*

PEG precipitates of supernatants of PVM, or mock infected (MI) cells labelled with [^{35}S]-methionine and lysates of these cells were analysed by SDS-PAGE. The samples were prepared as described in Section 2.5.12. Cell lysate from the equivalent of 5×10^5 BS-C-1 cells or PEG precipitate equivalent to supernatant from 2×10^6 BS-C-1 cells was used in each gel well. A multiplicity of infection of 1 was used and the labelling carried out at $15 \mu\text{Ci}$ [^{35}S]-methionine per ml for a 48 hour labelling period in the presence of actinomycin D ($2.5 \mu\text{g}$ per ml). Two prints of a fluorograph of PVM polypeptides analysed on a 15 per cent resolving gel are shown to enable bands of different intensities to be identified. The polypeptides are indicated by circles to the right of the tracks.

PEG Polyethylene glycol precipitate of cell supernatant.

PVM Samples from PVM infected cultures

MI Samples from mock infected cultures



precipitated supernatant sample.

It was possible that the cell type or the labelling conditions could affect the presumptive PVM polypeptides observed. Figure 5 shows PVM induced polypeptides synthesized in infected BS-C-1 and BHK-21 cells. The cells were infected and labelled under the same conditions as described above for the samples shown in Figure 4 except that where indicated a 1 hour pulse labelling period, beginning 48 hours after infection, was used rather than a 48 hour continuous labelling regime. A single virus specific band (N, 39K or both) was resolved in the 40K region in infected BS-C-1 cells on this gel whereas two virus specific bands (N and 39K) were resolved in the infected BHK-21 cells in this region. Two virus specific bands were sometimes resolved in this region in infected BS-C-1 cells but never as clearly as in BHK-21 cells. The pattern of polypeptides with low M_r s differed between the two cell types and also between different preparations of PVM infected BS-C-1 cell lysates. A 20K polypeptide was observed only when a 1 hour labelling period was used whereas a 18K polypeptide was observed only when a 48 hour labelling period was used. The variability in the pattern of polypeptides with low M_r s observed in cell lysates in Figure 5 is difficult to explain since the same virus stock was used at the same m.o.i. for each labelling period. In subsequent experiments (Section 3.3) no difference in the polypeptides immunoprecipitated from lysates of cells labelled for either 10 minutes or 24 hours (except for the absence of the 12K polypeptide after a

Figure 5. PVM polypeptides synthesized in PVM infected

BS-C-1 and BHK-21 cells

Cells were infected and labelled in a similar manner to that described in the legend to figure 4. Where 1 hour labelling periods were used these were carried out 72 hours after infection using 50 μ Ci per ml of [35 S]-methionine in the presence of actinomycin D (2.5 μ g per ml. The fluorograph shows PVM polypeptides observed in cell lysates when labelled under different conditions and analysed on a 6-15 per cent gradient gel. The polypeptides are indicated by circles to the right of the tracks.

Top line of figure heading indicates cell type infected.

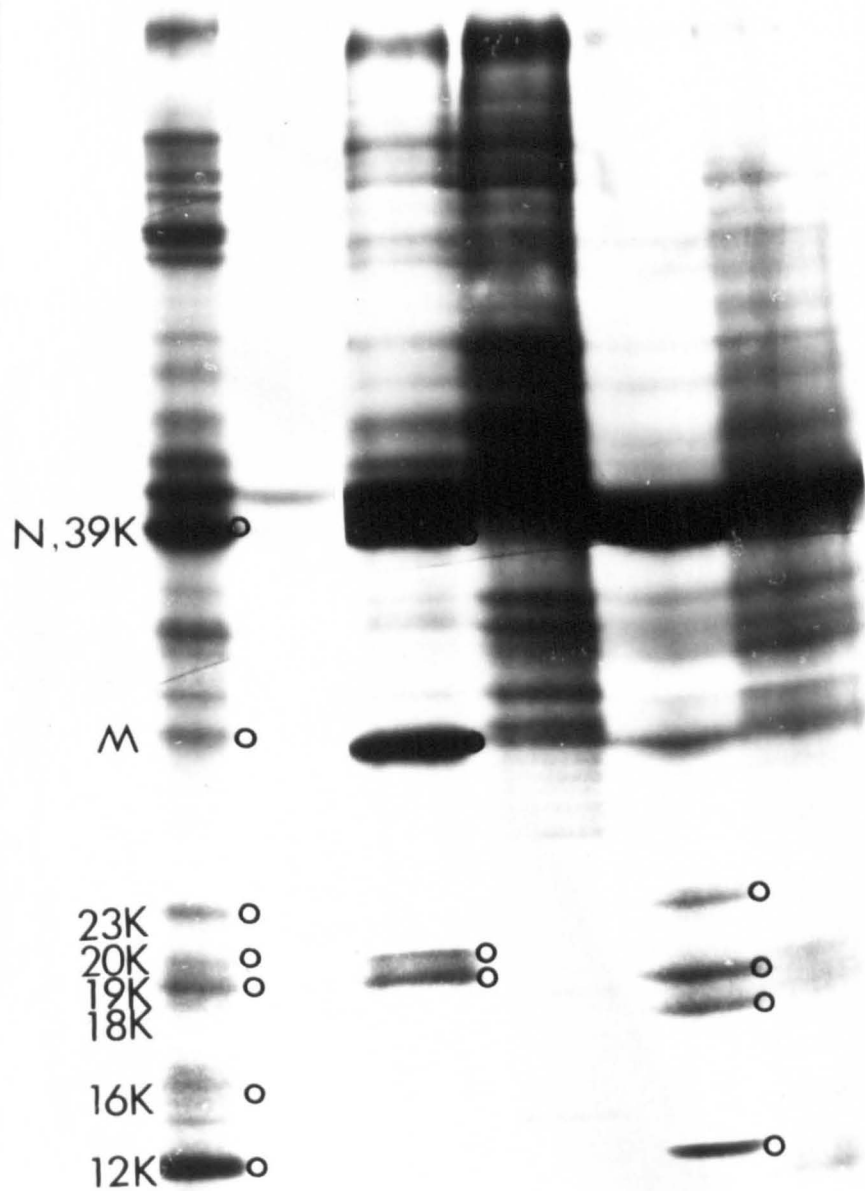
Middle line indicates duration of labelling.

Bottom row indicates whether cells were infected with PVM (PVM) or mock infected (MI).

BS-C-1
1 hour
PVM MI

BHK-21
1 hour
PVM MI

BS-C-1
48 hours
PVM MI



10 minute pulse) was observed and although no protease inhibitors were used in the experiment shown in Figure 5 this again was subsequently shown to have no effect on virus specific polypeptides with low M_r s (Section 3.3.11). The differences may have been due to different amounts of protein being loaded onto the gel. This result suggests that care needs to be taken when looking at virus induced polypeptides in total cell lysates. In contrast the pattern of polypeptides observed in experiments where immunoprecipitation was used (described in Section 3.1.3) to identify virus specific polypeptides was consistent although the possibility that not all virus specific polypeptides were recognized by the antiserum used has to be considered.

The failure to detect the G1, G2, and F1 polypeptides in the PVM infected cell lysates was probably due to poor labelling of these polypeptides and the high background of cellular polypeptides since in later experiments, (see Section 3.1.5), these polypeptides were detected by immunoprecipitation.

3.1.3. Analysis of PVM polypeptides using two dimensional polyacrylamide gel electrophoresis

Polyethylene glycol precipitates of the supernatants of radiolabelled virus infected cells were analysed by two dimensional polyacrylamide gel electrophoresis (Figure 6). The first dimension used non-equilibrium pH gradient electrophoresis to separate the polypeptides on the basis of

charge (O'Farrell *et al.* 1977) whereas the second dimension was SDS-PAGE to separate the polypeptides primarily on the basis of size. The acidic end is on the right. Diagrams are shown adjacent to the fluorographs of the PVM and RS virus samples to aid the identification of virus specific spots. The pattern of spots obtained on analysis of the PEG precipitate of the supernatant of RS virus infected cells showed the N, P and 15K polypeptides to be relatively acidic whereas the M and M2 polypeptides were relatively basic (Figure 6a). Several polypeptides with the same mobility as the M protein in the second dimension but which were slightly more acidic could also be seen. A possible explanation of what these spots may represent is considered in Section 4. Two virus specific polypeptides (labelled N and M) could be identified in the PEG precipitates of proteins in the supernatants of PVM infected cells by comparing the pattern of spots observed in Figure 6b with that observed with PEG precipitates of the supernatants of uninfected cells, (Figure 6c). The M polypeptide co-migrated with a host cell protein but the spot showed a higher intensity relative to the other spots when compared with the pattern obtained with supernatants of uninfected cells. The M polypeptide was observed to be basic. The N polypeptide was observed to be acidic but to have streaked in the direction of the separation carried out on the basis of charge. This may have been due to charge heterogeneity or, more likely a combination of aggregation as indicated by the large amount of label at the acid end (where the sample was

Figure 6. Analysis of PVM and RS virus polypeptides in PEG precipitates of infected cell supernatants on two dimensional gels.

Pneumovirus infected or mock infected cells (5×10^4) were labelled from 48 to 72 hours post-infection with [^{35}S]-methionine at 1mCi per ml in the presence of actinomycin D ($2.5 \mu\text{g}$ per ml). PEG precipitates of the supernatants of these cells were analysed by non-equilibrium pH gradient electrophoresis followed by SDS-PAGE as described in Section 2.5.17. NEPHGE was carried out in the horizontal direction with the acid end on the right. Diagrams are shown adjacent to the fluorographs of PVM and RS virus polypeptides to aid the interpretation of the data.

a) Fluorograph of PEG precipitate of RS virus infected cell supernatant analysed by NEPHGE/SDS-PAGE

b) Fluorograph of PEG precipitate of PVM infected cell supernatant analysed by NEPHGE/SDS-PAGE

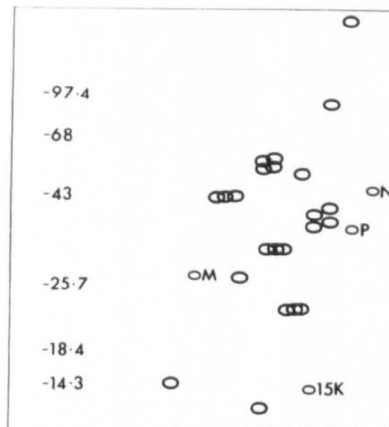
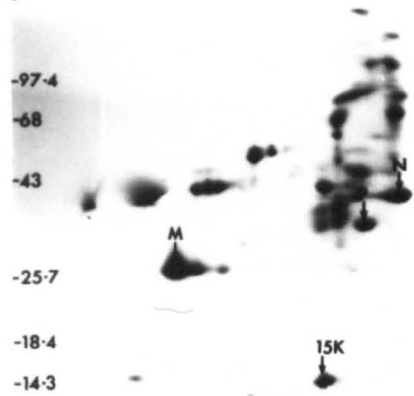
c) Fluorograph of PEG precipitate of mock infected cell supernatant analysed by NEPHGE/SDS-PAGE

BASIC

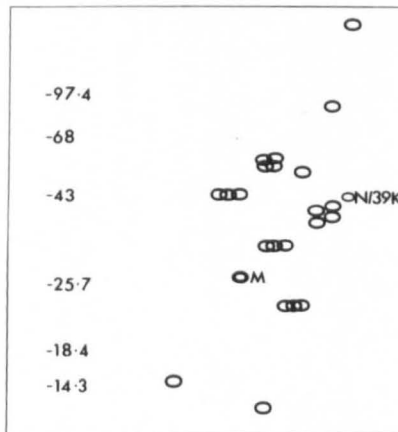
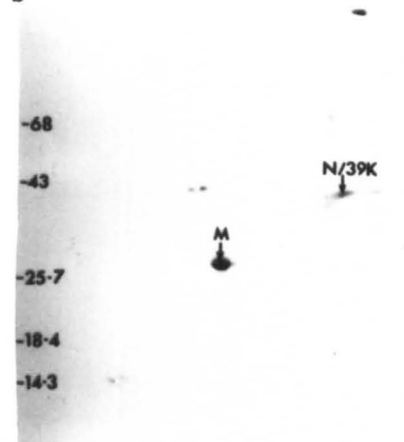
← NEPHGE

ACIDIC

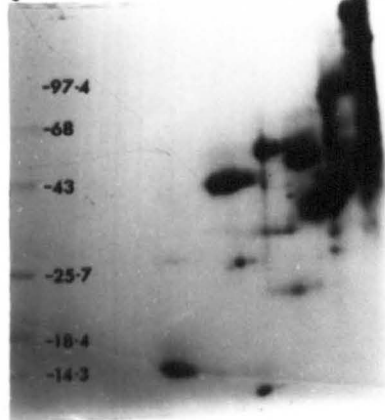
a



b



c



SDS-
PAGE



loaded) and association with nucleocapsid structures.

Figure 7 shows lysates of [^{35}S]-methionine labelled BS-C-1 cells infected with RS virus (a), PVM (b), or uninfected (c), separated on two dimensional gels. The positions of virus specific polypeptides superimposed on the polypeptide pattern of uninfected cells are shown adjacent to each fluorograph. It can be seen that each virus had acidic polypeptides with M_r s in the regions of 40K, 35K and 16K and a more basic polypeptide of about 30K. RS virus infected cell lysates revealed four spots in addition to the four spots shared with other members of the *Pneumovirus* genus (Figure 7a). A diffuse region of label in the 45K region probably corresponded to the F1 part of the fusion protein. The M2 polypeptide and two higher mobility polypeptides (with M_r s of 20K and 17K on this gel) were highly basic. The 20K polypeptide can be seen more clearly on the longer exposure of this gel shown in Figure 63.

There were several PVM specific spots on the autoradiograph of PVM polypeptides analysed on a two dimensional gel (Figure 7b). Two basic spots in the 45K to 50K region probably represented two forms (F0 and F1) of the major polypeptide expressed on the surface of infected cells which is described in Section 3.3.13. An additional spot below the nucleocapsid streak corresponded to the 39K polypeptide and two acidic spots occurred in the region of the 25K band which was usually observed as a broad smear on single dimension gels (Figure 4). Three acidic polypeptides corresponding to the 20K, 19K and 18K polypeptides observed

Figure 7. Analysis of PVM and RS virus polypeptides in infected cell lysates on two dimensional gels.

BS-C-1 cells were infected and labelled as described in the legend to Figure 6. The cells were lysed and analysed by non-equilibrium pH gradient electrophoresis followed by SDS-PAGE as described in section 2.5.17. NEPHGE was carried out in the horizontal direction with the acidic end to the right. Diagrams of the virus specific polypeptides overlaid on the mock infected cell polypeptides are shown adjacent to each autoradiograph.

- a) Autoradiograph of gel of RS virus infected cell lysate.
- b) Autoradiograph of gel of PVM infected cell lysate.
- c) Autoradiograph of gel of mock infected cell lysate.

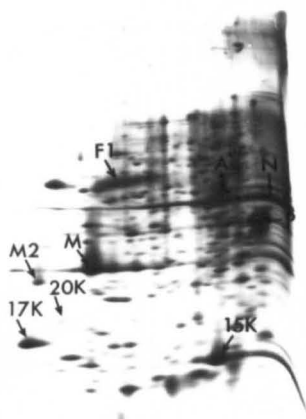
BASIC

← NEPHGE

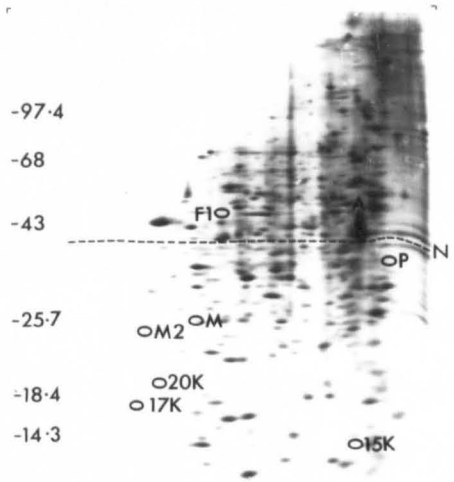
ACIDIC

a

-97.4
-68
-43
-25.7
-18.4
-14.3

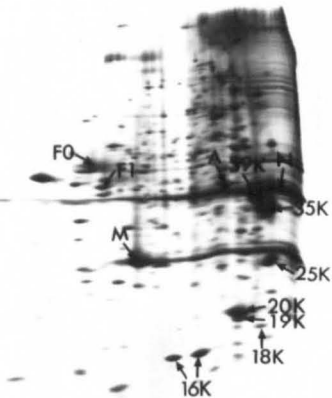


-97.4
-68
-43
-25.7
-18.4
-14.3

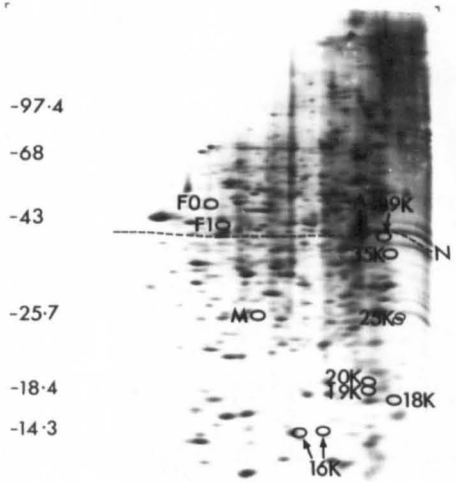


b

-97.4
-68
-43
-25.7
-18.4
-14.3

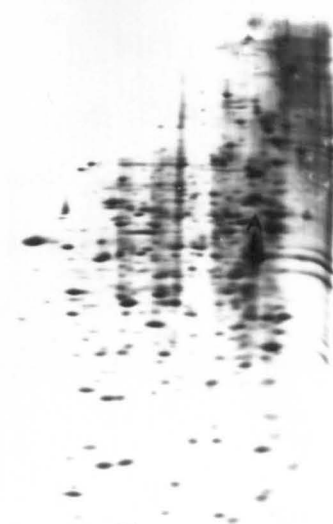


-97.4
-68
-43
-25.7
-18.4
-14.3



c

-97.4
-68
-43
-25.7
-18.4
-14.3



SDS-PAGE



single concentration gels were observed. Two slightly acidic spots were observed with mobilities corresponding to a 16K polypeptide.

PVM and RS virus had five polypeptides with similar mobilities and charges (F1, N, P/35K, M and 15K/16K). Since these were the strongest spots observed on two dimensional gels of the polypeptides from PVM or RS virus infected cells they were probably the most abundant methionine containing polypeptides. The F1 polypeptides were basic, and produced rather diffuse spots streaked in the direction of the charge separation. The N polypeptides did not resolve well in the first dimension of any of the gels described above. This could have been due to association of the polypeptides with RNA. The P and 35K polypeptides were observed to be acidic. In the case of RS virus the P protein is known to be phosphorylated (Cash et al. 1979b). Phosphate groups are not responsible for the acidic nature of the PVM 35K polypeptide since it does not appear to be phosphorylated. The M polypeptides of RS virus and PVM were observed to be basic suggesting that these polypeptides may be homologous. Although both viruses had polypeptides migrating slightly faster than the M polypeptide the 25K polypeptide of PVM was observed to be acidic suggesting that it had different properties from the corresponding polypeptide (M2) observed in one dimensional separations of RS virus polypeptides which is highly basic (Dubovi 1982, Figure 7b). The 15K polypeptide of RS virus (possibly representing the 14K non-structural protein or one of the 16K polypeptides

synthesized *in vitro*) and the two 16K polypeptides of PVM were acidic.

3.1.4 Immunoprecipitation of [³⁵S]-methionine labelled PVM and RS virus polypeptides

Immunoprecipitation of RS virus and PVM polypeptides was performed to confirm the virus specificity of the polypeptides described in the preceding sections. The anti-PVM serum used was a murine anti-PVM serum obtained during the production of monoclonal antibodies to this virus. The mice had been immunized with PVM infected Balb/c ₃T₁₂ cells and so the antiserum was expected to contain antibodies to non-structural as well as structural polypeptides of the virus. Since Balb/c mice were used little antibody to host cell polypeptides would be expected to be produced and this would not necessarily react with BS-C-1 cell proteins. In some of the later experiments described in Section 3.3 this serum was adsorbed using ten per cent w/v acetone fixed BS-C-1 cells and this removed most antibody to host cell proteins. The anti-RS virus serum was obtained from Dr. E.J. Stott (AFRC, Compton) and had been raised in gnotobiotic calves.

The polypeptides precipitated from radiolabelled cell lysates and PEG precipitates of the supernatants of radiolabelled cells using the antisera described above and formalin fixed *S.aureus* are shown in Figure 8. The samples from PVM infected and mock infected cells were

**Figure 8. PVM and RS virus polypeptides immunoprecipitated
by homologous antisera**

Cell lysates and polyethylene glycol precipitates of the supernatants from 1×10^6 PVM and RS virus infected BS-C-1 cells labelled from 48 to 72 hours post infection ($50 \mu\text{Ci}$ [^{35}S]-methionine per ml) were immunoprecipitated with the homologous antisera described in the text and analysed by SDS-PAGE. The fluorograph shows the polypeptides immunoprecipitated analysed on a 6-15 per cent gradient gel.

PEG PEG precipitate of infected cells

immunoprecipitated with homologous antisera.

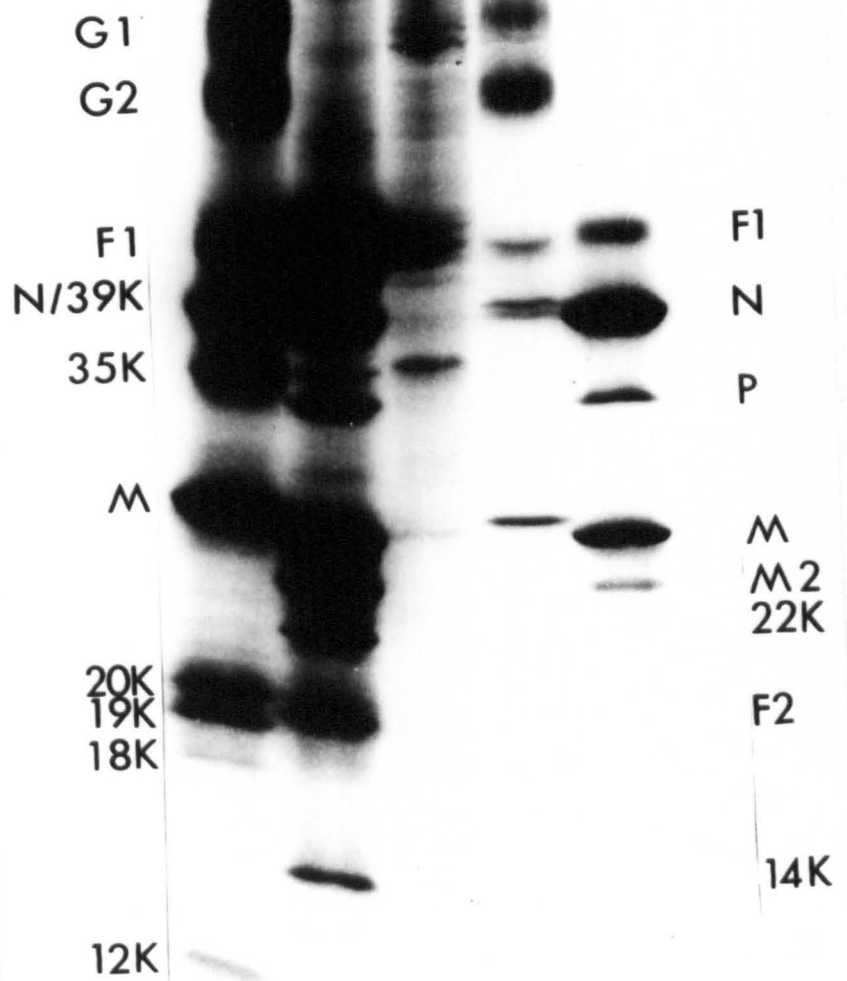
MI Mock infected cell samples that were

immunoprecipitated with the anti-PVM serum.

PVM polypeptide designations shown on the left and

RS virus polypeptide designations on the right.

CELL LYSATE PEG
PVMRS MIPVM RS MI



immunoprecipitated using the anti-PVM serum whereas the anti-RS virus serum was used to precipitate polypeptides from the samples from RS virus infected cells. The F1, N, P, M, M2, F2 and 14K polypeptides of RS virus were readily identified whereas the identity of the 22K polypeptide was less clear. The 22K polypeptide may represent a different form of M2 since several forms of this polypeptide with different mobilities have been described (Routledge et al., 1987a). The 22K and F2 polypeptides were observed in the PEG precipitate track on overexposure of the gel unlike the 14K polypeptide suggesting that they were structural polypeptides. The L polypeptides of both RS and PVM were not observed on the gel shown in Figure 8 but were observed on other gels (e.g. Figure 39).

The major PVM specific polypeptides shown in Figure 8 were reproducibly precipitated by the anti-PVM antiserum. G1 was usually a more intense band than G2 in cell lysates whilst the reverse was observed to be the case in the PEG precipitate. The N polypeptide and a 39K polypeptide with a slightly faster mobility than the N polypeptide could not always be resolved from each other. The 35K polypeptide could only be identified when immunoprecipitation was used and appeared to be absent from the supernatant sample. The M polypeptide was always observed but the 25K polypeptide was not. The reason for this was not clear since the antiserum did immunoprecipitate bands corresponding to such a polypeptide from PVM polypeptides synthesized *in vitro* (Figure 11). Cash, 1979 identified at least 2 bands in this

region on under exposure of a gel. The 20K, 19K and 12K polypeptides were reproducibly immunoprecipitated by the anti-PVM serum and were identified in immunoprecipitates of PEG samples if the fluorographs were exposed for a sufficient period of time (e.g. Figure 12). The 18K polypeptide was not always observed and in some cases a 16K polypeptide was observed (Figure 12).

The polypeptides designated G1, G2, N, 39K, M and 25K are probably equivalent to polypeptides II (M_r of 79,000, 79K), III (63K), IV (42K), V (40K), VII (28K) and VIII (26K) previously described by Cash *et al.* (1979a), since they showed similar relative mobilities. However, there were several differences from the pattern described by Cash *et al.* (1979a) which included the polypeptide designated F1, the absence of the 200K and 35K polypeptides when immunoprecipitation was not used and a larger number of polypeptides with low M_r s which could be virus specific (Table 7). The absence of polypeptides of about 200K and 35K in Figures 4 and 5 was probably due to poor label incorporation and/or high background incorporation into cellular polypeptides, because polypeptides with calculated M_r s of 200K and 35K were observed when the samples were immunoprecipitated. The 20K and 19K polypeptides were probably genuine virus specific polypeptides since they were immunoprecipitated by anti-PVM serum, and are probably equivalent to polypeptide IX (17K) described by Cash *et al.* (1979a). This suggestion was made on the basis that the polypeptide designated IX by these authors had a similar

mobility to the 20K and 19K polypeptides that were observed during the course of this project and were not always well resolved. The 23K, 18K and 16K polypeptides were usually weak bands and their virus specificity appears uncertain although their intensity may simply reflect the labelling conditions. The 18K polypeptide had a similar relative mobility to polypeptide X, (16K), described by Cash *et al.* (1979a). The 12K band had a similar relative mobility to polypeptide XI, (13.5K), identified by Cash *et al.* (1979a). No band equivalent to polypeptide XII, a 12K non-structural polypeptide described by Cash *et al.* (1979a), was ever observed. The 9K polypeptide observed in Figure 4 was not often observed and its significance is not clear, a possible explanation of what this band may represent is discussed later (3.3.15). When immunoprecipitation was used polypeptide G2 as well as polypeptide G1 could be observed in PVM infected cell lysates in contrast to the results of Cash *et al.* (1979a) who only observed G1 in infected cell lysates. The reason for this was probably that G2 was not well labelled and could not be detected above the background of labelled cellular polypeptides by Cash *et al.* (1979a), whereas the use of immunoprecipitation enabled the polypeptide to be precipitated from a larger number of cells than could be lysed and run on a single gel track. Immunoprecipitation also reduced the background of labelled cellular polypeptides that were observed. The 25K polypeptide, (polypeptide VIII of Cash *et al.* 1979a), was not observed in experiments involving immunoprecipitation

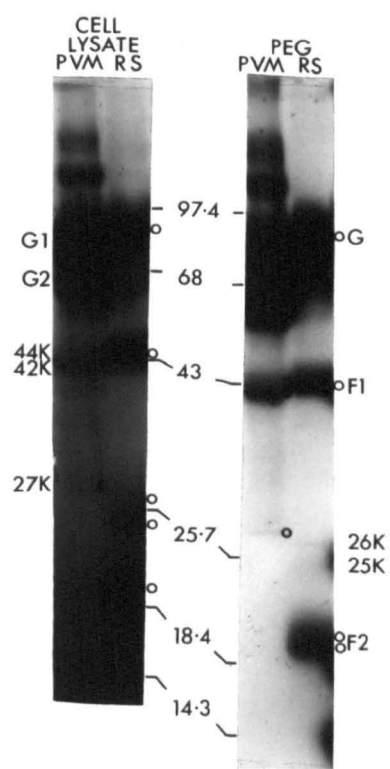
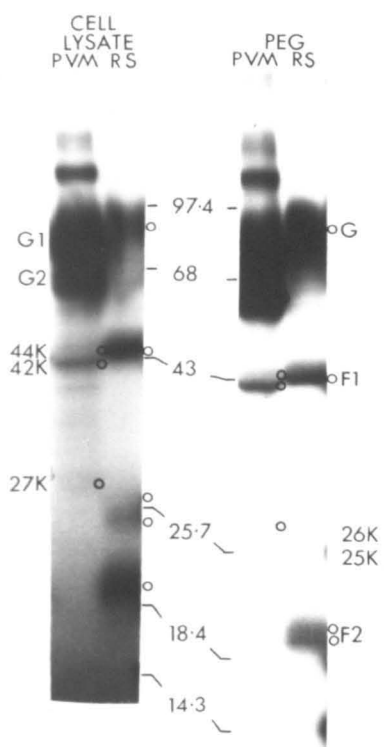
which could have been due to low levels of antibody against this polypeptide, although as mentioned above the antiserum precipitated this polypeptide from among the products of *in vitro* translation reactions, or the polypeptide may not have been present in the samples at sufficient level to detect. The 35K polypeptide was observed to be non-structural as described by Cash et al. (1979a). The 18K polypeptide (polypeptide X) was observed in the PEG precipitate after immunoprecipitation in contrast to the results of these authors who did not use immunoprecipitation. This discrepancy was probably due to the greater sensitivity that could be achieved using immunoprecipitation enabling polypeptides of low abundance to be detected.

3.1.5. Immunoprecipitation of [³H]-glucosamine labelled PVM and RS virus polypeptides

Figure 9 shows the [³H]-glucosamine labelled polypeptides that were immunoprecipitated by the anti-PVM and anti-RS virus antibodies. The anti-RS virus serum immunoprecipitated the G, F1 and F2 polypeptides along with 26K and 25K polypeptides from both cell lysates and PEG precipitates of [³H]-glucosamine labelled, RS virus infected BS-C-1 cells. The F2 polypeptide could be resolved into two bands in the PEG precipitate sample. The G protein was identified in immunoprecipitates of [³H]-glucosamine labelled but not of [³⁵S]-methionine or [³H]-leucine labelled RS virus cell lysates or PEG precipitates. This has been observed by others and is considered to be due to low

Figure 9. [³H]-glucosamine labelled PVM and RS virus polypeptides immunoprecipitated with homologous antisera

PVM and RS virus infected cells were infected, labelled and immunoprecipitated as described in the legend to Figure 8 except that [³H]-glucosamine was used to label the cells instead of [³⁵S]-methionine. The fluorograph shows the labelled polypeptides immunoprecipitated analysed on a 6-15 per cent gradient gel. The labelling of the figure is the same as described for Figure 8.



abundance, low methionine content and high glucosamine content of this polypeptide.

The anti-PVM serum immunoprecipitated G1, G2 a 42K polypeptide and possibly also 44K and 27K bands from PVM infected, cell lysates and PEG precipitates of the same cultures. The nature of the 44K and 42K polypeptides is considered further in Section 3.3. The 27K band was not usually observed and probably represented a co-precipitating host cell polypeptide or was due to a small fraction of the ^3H becoming transferred from carbohydrate to amino acids. The bands observed above G1 in the figure were not virus specific (see Figure 29).

The glycosylated 26K and 25K RS virus specific polypeptides identified by immunoprecipitation were probably related to the F protein since at least one polypeptide with a similar mobility to these polypeptides was immunoprecipitated by an anti-F but not by an anti-G monoclonal antibody (Figure 47). The band labelled F2 was considered to be the F2 protein since it was the most heavily labelled of the F related polypeptides and the F2 subunit of the RS fusion protein has 4 of the 5 potential glycosylation sites (Collins et al., 1984b; Baybutt & Pringle, 1987). The two bands observed in the region of the gel where F2 occurred in the immunoprecipitate of the PEG precipitated supernatant may have been due to variations in the extent of glycosylation of the F2 polypeptide. The other F related polypeptides could be fragments of F1.

3.1.6. PVM polypeptides identified on Western blots

The polypeptides of PVM that could be identified on Western blots of PVM infected cell lysates and PEG precipitates of the supernatants of PVM infected cells using the murine anti-PVM serum are shown in Figure 10. The pattern of polypeptides observed differed from that seen in immunoprecipitates and varied between samples (compare Figure 10a and c). Possible explanations for this are considered below. Polypeptides N, 39K, 35K, M and 18K were usually observed whereas the occurrence of the 25K, 23K and 20K polypeptides was more variable. Figure 10b shows the absence of a reaction when blots were incubated with pre-immune mouse serum instead of anti-PVM serum.

The variation in the pattern of polypeptides observed could have been due to different amounts of sample being loaded on the gel, different transfer efficiencies or the use of different preparations of anti-PVM serum. The presence of a 35K polypeptide in PEG precipitates could have been due to the relatively high level of PEG precipitate loaded on the gels (obtained from 5 times the number of cells used to prepare the cell lysate in the adjacent tracks). The band may be due to low levels of 35K polypeptide, possibly contamination from disrupted cells, or another protein of similar mobility on the gel that cross reacts with the anti-PVM serum. Failure to detect G2 could have been due to the presence of cellular proteins with which the antiserum cross reacted in this region of the gel. Failure to detect G1, G2, F1, 12K and sometimes other

Figure 10. PVM polypeptides identified on Western blots

Polypeptides of PVM or mock infected cell lysate (equivalent to 5×10^5 cells) and PEG precipitates of supernatants from 2×10^6 PVM infected or mock infected BS-C-1 cells were separated by SDS-PAGE and transferred to nitrocellulose as described in Section 2.5.18. The nitrocellulose was stained to show the positions of the tracks and markers, destained, blocked and the polypeptides recognized by the antisera visualised as described in Section 2.5.19.

a) Blot reacted with anti-PVM serum.

The designations of the PVM specific polypeptides are indicated on the left.

PEG PEG precipitates

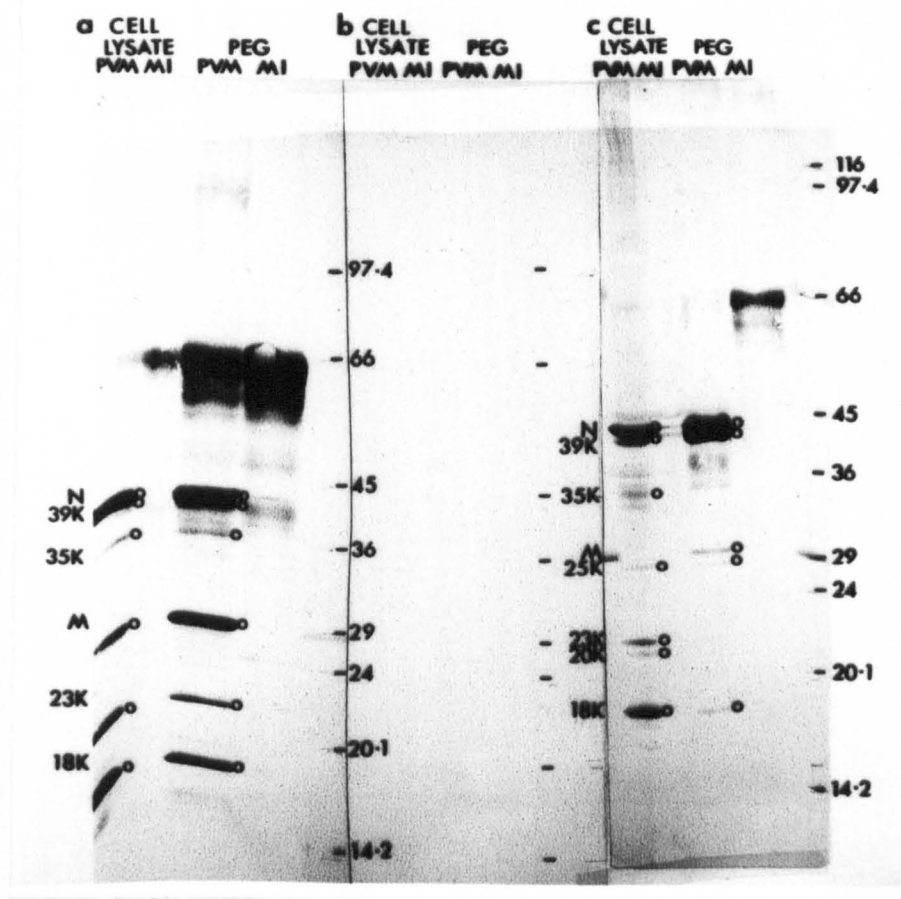
PVM PVM infected cell samples

MI Mock infected cell samples

b) Blot reacted with pre-immune mouse serum.

c) Blot reacted with anti-PVM serum.

(b) and (c) are labelled in the same way as (a)

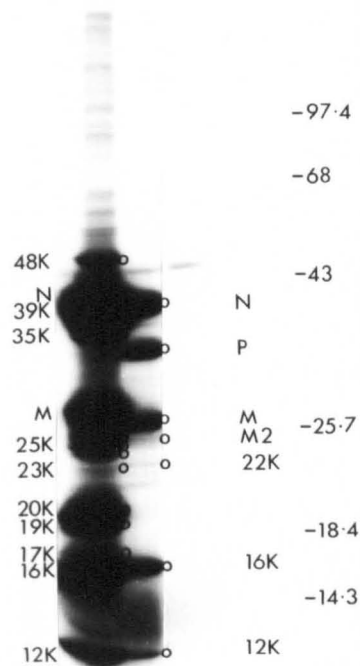


polypeptides may have been due to low abundance of the polypeptide, failure of the polypeptide to transfer from the gel to the nitrocellulose or inability of the antibodies present in the serum to recognize some of the polypeptides when denatured. It is also possible that some of the polypeptides identified in immunoprecipitation experiments were only detected due to co-precipitation with other polypeptides and that the serum did not actually contain antibodies directed against them. Immunoprecipitation appeared to give more consistent results and since this was necessary for some experiments, for example pulse chase experiments, this was the method used for identifying virus specific polypeptides in most of the experiments described in this thesis.

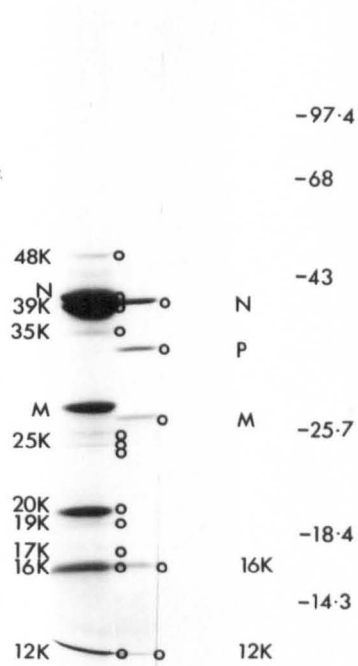
3.1.7 PVM and RS virus polypeptides synthesized *in vitro*

The polypeptides synthesized *in vitro* using RNA from PVM and RS virus infected and mock infected cells were analysed by SDS-PAGE without prior immunoprecipitation (Figure 11). Polypeptides corresponding to the N, 39K, 35K, M, 20K, 19K, 16K and 12K polypeptides observed *in vivo* were observed among the *in vitro* translation products obtained using RNA from PVM infected cells. A 48K polypeptide synthesized *in vitro* may correspond to an F related polypeptide, (F0 and F1 both occur in this region of the gel and neither was extensively glycosylated, Figure 38). Three bands observed below M in Figure 12 (labelled 25K) probably correspond to the 25K polypeptide described in Section 3.1.2

a PVM RS MI



b PVM RS MI



and the 24K polypeptide described in Section 3.1.9. There was a weak 23K polypeptide band the significance of which was unclear since there was a greater degree of incorporation of label into many polypeptides in this track compared with the mock infected RNA track. The relationship of the 17K polypeptide to PVM polypeptides labelled *in vivo* is not known. Fewer polypeptides could be identified amongst the *in vitro* translation products of RNA from RS virus infected cells. N, P, M, some bands possibly corresponding to different forms of M2 and a 16K polypeptide could be clearly identified with possibly a faint 22K polypeptide similar to the 23K polypeptide observed with PVM and a 12K polypeptide.

Most of the unglycosylated PVM and RS polypeptides identified *in vivo* could therefore be identified amongst the *in vitro* translation products obtained using RNA from virus infected cells. The L polypeptide of PVM or RS was not detected amongst the *in vitro* translation products probably due to their large size. A polypeptide that could correspond to the PVM L protein immunoprecipitated from the *in vitro* translation products obtained using RNA from PVM infected cells is, however, described below (Section 3.1.8). The PVM glycoproteins were not identified amongst the *in vitro* translation products of RNA from PVM infected cells as would be expected, but the 48K polypeptide probably represented an unglycosylated form of one of them since it was a relatively large polypeptide that was not observed *in vivo*. The 14K polypeptide of RS virus and the two glycoproteins were not

identified among the *in vitro* translation products of RNA from RS virus infected cells. If the 14K polypeptide was poorly labelled failure to detect it could have been due to it being obscured by the globin band (the broad smear near the bottom of the gel due to translation of residual globin mRNA in the reticulocyte lysate). The absence of any detectable glycoprotein precursors could have been due to the low abundance of these messages and, in the case of the G glycoprotein, the low methionine content of the polypeptide. The identity of the 16K and 12K polypeptides observed amongst the *in vitro* translation products was not clear. The 12K polypeptide may have represented the 11K non structural polypeptide but the mobility of the 16K polypeptide appeared to be too low for it to represent the 14K polypeptide. Aberrant mobility did not appear to be a likely explanation for this band since a 14K polypeptide was observed *in vivo*.

3.1.8. Immunoprecipitates of PVM and RS virus polypeptides synthesized *in vitro*

The polypeptides synthesized *in vitro* and immunoprecipitated by the antisera described in Section 3.1.4 are shown in Figure 12. The polypeptides immunoprecipitated from infected cells and PEG precipitates of supernatants of such cells labelled *in vivo* are also shown in Figure 12 for comparison. Two exposures of the gel are shown so that all the polypeptides observed can be seen. The polypeptide patterns observed in this figure are similar

Figure 12. PVM and RS virus polypeptides synthesized *in vitro* and immunoprecipitated

PVM and RS virus polypeptides synthesized *in vitro* as described in the preceding figure were immunoprecipitated and analysed by SDS-PAGE using a 6-15 per cent gradient resolving gel. The polypeptides immunoprecipitated from samples labelled *in vivo* were run on the same gel for comparison and the gel was fluorographed. Two exposures of the fluorograph are shown to enable bands of different intensities to be identified. The antisera used were murine anti-PVM serum (anti-PVM) and bovine anti-RS serum (anti-RS).

PEG Polypeptides immunoprecipitated from PEG precipitates of cell supernatants

PVM Samples derived from PVM infected cells or RNA from such cells translated *in vitro*

RS Samples derived from RS virus infected cells or RNA from such cells translated *in vitro*

MI Samples derived from mock infected cells or RNA from such cells translated *in vitro*

The PVM specific polypeptides are labelled on the left and the RS virus specific polypeptides on the right. The polypeptides are indicated by circles and the RS polypeptides by r's on the right of each track.

to those observed in the previous figure but the polypeptides with lower M_r s are reduced in intensity or absent presumably due to there being lower levels of antibody directed against these smaller polypeptides than against the larger ones. The immunoprecipitate of PVM polypeptides labelled *in vitro* revealed a polypeptide with a high M_r migrating at a similar position to polypeptide L labelled *in vivo*. Some of the PVM polypeptides labelled *in vitro* were observed in the track immunoprecipitated with anti-RS serum. This may have represented non-specific binding since the intensity of the bands was very low compared with that of the polypeptides immunoprecipitated with the homologous antiserum or may have been due to the viral polypeptides sharing common epitopes. Since pre-immune serum was not used in this experiment it is not possible to distinguish between these possibilities. The 48K, N, 35K, M, 25K, 20K and two bands in the region of the 16K polypeptide were immunoprecipitated from the *in vitro* translation products of RNA from PVM infected cells. The 19K polypeptide of PVM and the 12K polypeptides of PVM and RS virus were absent in contrast to the situation when immunoprecipitation was not used (Figure 11). This suggested that the 19K and the 12K polypeptides that were observed in immunoprecipitates of PVM polypeptides labelled *in vivo* may have been precipitated due to association with other polypeptides rather than because they were recognized by antibody. This is in agreement with the results obtained in Western blotting experiments where the 19K and 12K

polypeptides were not recognized by antibody (Figure 10). An alternative explanation would be that the antibody recognizes epitopes absent on the polypeptides synthesized *in vitro* and on denatured polypeptides.

3.1.9 Analysis of PVM and RS virus polypeptides synthesized *in vitro* on two dimensional gels

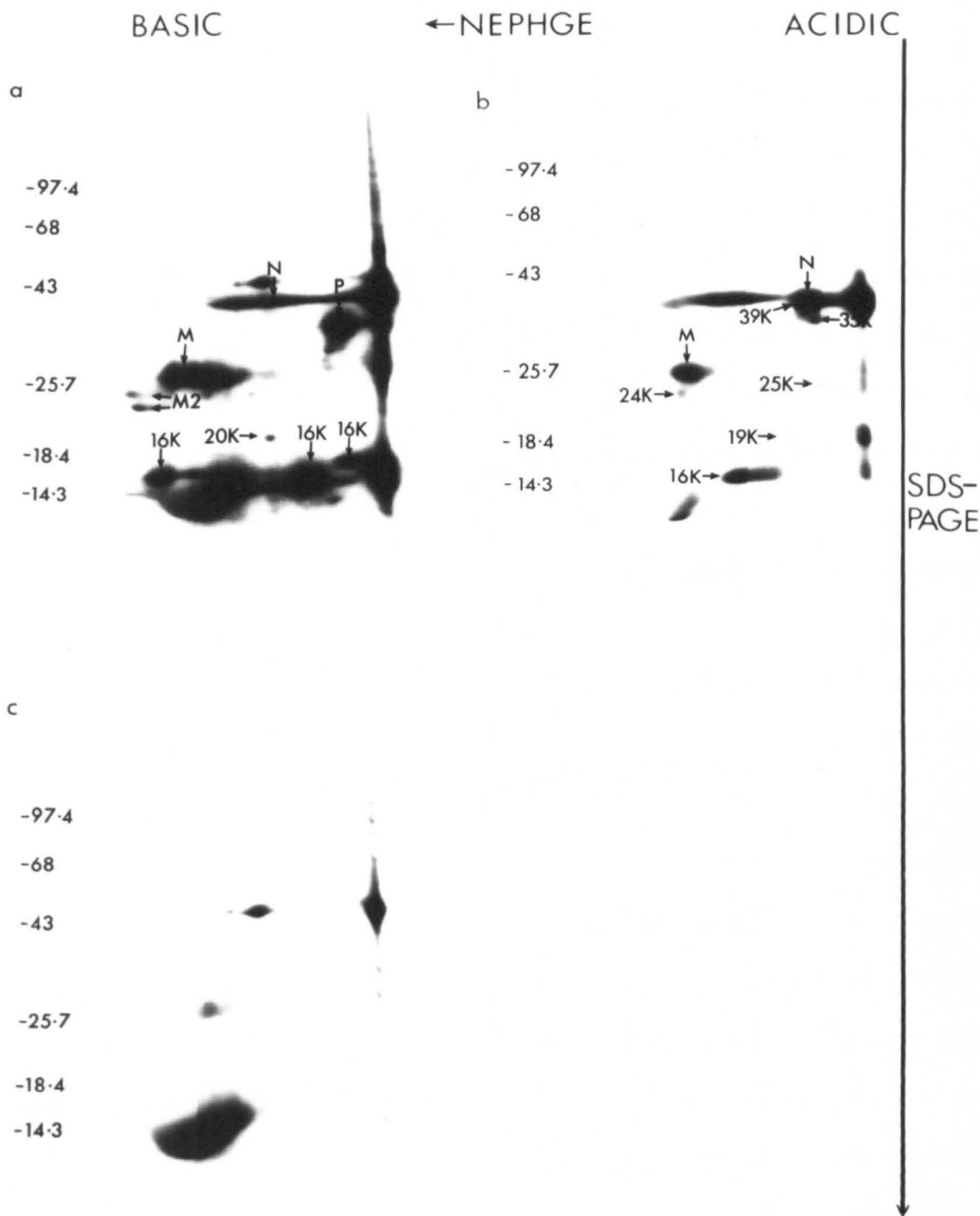
The polypeptides synthesized *in vitro* were analysed on two dimensional gels without prior immunoprecipitation (Figure 13). The RS virus polypeptides synthesized *in vitro* (Figure 13a) included the N, P, M and M2 polypeptides identified amongst the RS virus polypeptides synthesized in RS virus infected cells (Figure 7). A 20K polypeptide observed amongst the polypeptides synthesized *in vitro* was more acidic than the polypeptide of similar mobility identified amongst RS virus polypeptides synthesized *in vivo*, suggesting that these polypeptides were different. The basic 16K polypeptide occurred in a similar position to a 17K polypeptide synthesized *in vivo* and these polypeptides were probably therefore equivalent. The other two polypeptides with mobilities corresponding to polypeptides with M_r s of about 16K were more acidic than the 16K polypeptide observed amongst the polypeptides synthesized *in vivo*.

The N, 39K, 35K, M, 25K and 19K polypeptides of PVM present in Figure 13b occurred in similar locations on the gel to polypeptides synthesized in PVM infected cells (Figure 7). A 24K basic polypeptide that had not been

Figure 13. Analysis of PVM and RS virus polypeptides synthesized *in vitro* on two dimensional gels.

Polypeptides synthesized *in vitro* as described in the legend to Figure 11 were analysed by NEPHGE/SDS-PAGE as described in Section 2.5.17. NEPHGE was carried out in the horizontal direction with the acidic end to the right.

- a) Fluorograph of PVM polypeptides synthesized *in vitro* analysed by NEPHGE/SDS-PAGE
- b) Fluorograph of RS polypeptides synthesized *in vitro* analysed by NEPHGE/SDS-PAGE
- c) Fluorograph of mock infected cell polypeptides synthesized *in vitro* analysed by NEPHGE/SDS-PAGE



observed among the PVM polypeptides synthesized in infected cells was present. The 19K spot was somewhat diffuse and may have represented the 20K and 19K polypeptides that were not well resolved. The 18K polypeptide was not observed. Two acidic 16K polypeptides were observed similar to those observed *in vivo* (Figure 7).

The N polypeptides of PVM and RS virus synthesized *in vitro* showed streaking in the first dimension as had been observed for this polypeptide synthesized *in vivo*. This was probably due to aggregation, (a large amount of label remained at the origin) and perhaps association with viral RNA. The matrix polypeptide of RS virus had several more acidic spots associated with it as was observed for this polypeptide synthesized *in vivo* and which was probably due to minor forms with different arrangements of disulphide bonds as described in Section 3.1.3. The basic 24K polypeptide of PVM has similar a similar electrophoretic mobility and charge properties to the M2 polypeptide of RS virus. The acidic 25K polypeptide of PVM observed both *in vitro* and *in vivo* did not have a counterpart amongst RS virus polypeptides. The 25K polypeptide may be related to the 39K polypeptide (Section 3.4.7). The nature of the 16K polypeptide of PVM is not clear. The nature of the 20K and 16K polypeptides observed amongst the products of *in vitro* translation of RNA from RS virus infected cells was not clear since they did not correspond to the polypeptides observed by others, (e.g. Collins et al. 1984a). The central 16K polypeptide may have corresponded to the 14K polypeptide

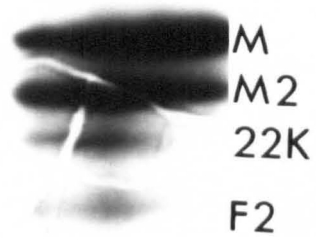
**Figure 14. Absence of cross reaction of anti-RS virus serum
with PVM polypeptides**

PVM (P), RS virus (R) and mock infected (M) cells were labelled and the cell lysates and PEG precipitates of the supernatants were immunoprecipitated with anti-RS virus serum and analysed by SDS-PAGE using a 6-15 per cent gradient gel. A fluorograph of the gel is shown. RS virus specific polypeptides are labelled on the right.

0.

anti-RS serum

Cell lysate		PEG		Cell lysate		PEG	
P	M	P	M	R		R	



observed amongst the polypeptides synthesized *in vivo*, the others possibly representing breakdown products of larger polypeptides or polypeptides prematurely terminated during synthesis.

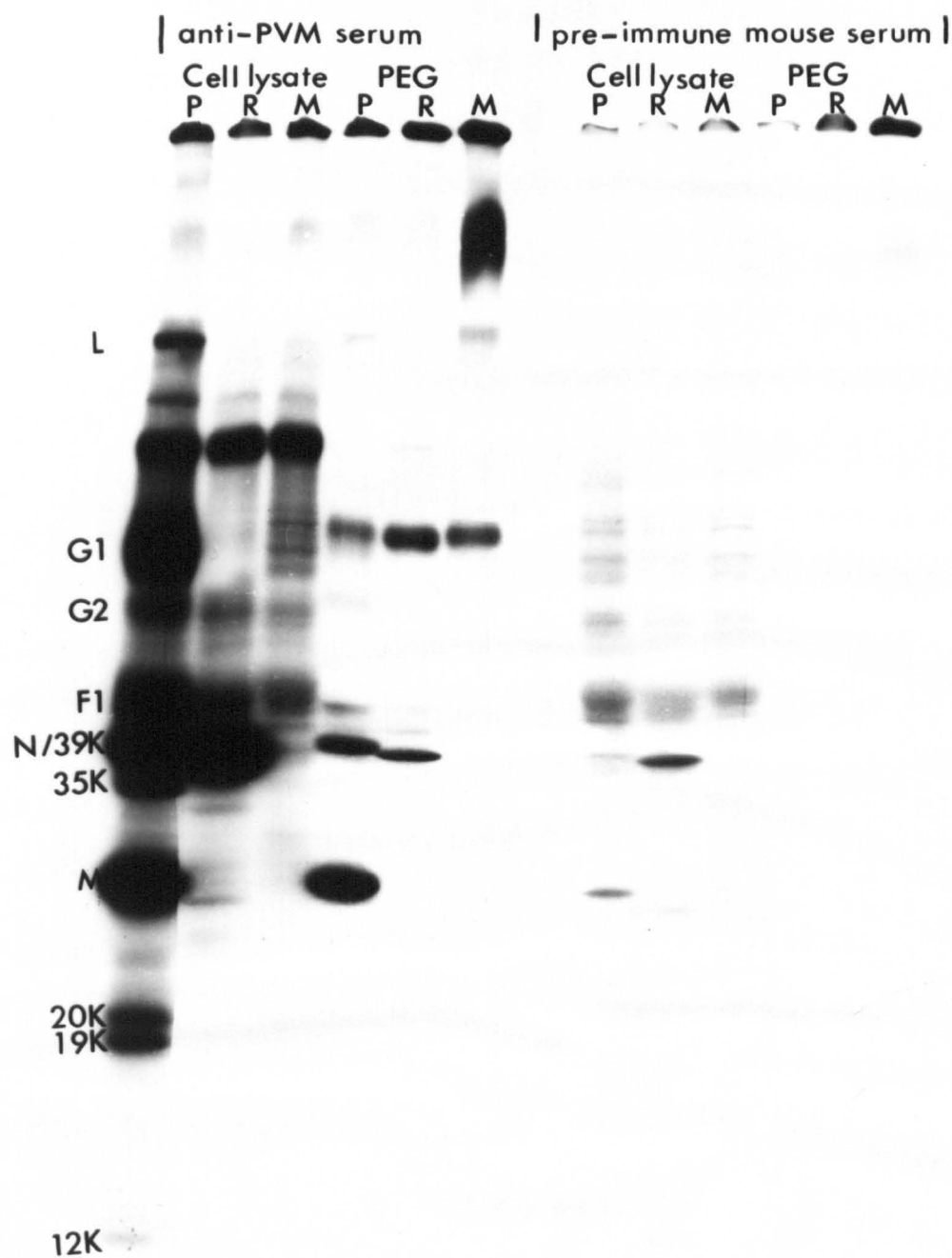
3.1.10 Cross-immunoprecipitation between PVM and RS virus polypeptides

Gimenez et al. (1984) observed a cross reaction on a Western blot of a murine anti-RS virus serum with the nucleocapsid protein of PVM. It was of interest to know if a similar cross reaction could be obtained using the murine anti-PVM serum produced during the present study. Since the Western blotting system was less well developed than the immunoprecipitation studies the latter system was used for examining the cross reaction of the antisera with heterologous virus. Western blotting was unsuitable primarily because the antibody system for detecting RS virus antigen did not give clear results so it was not possible to determine whether a negative reaction of anti-PVM serum with RS virus antigens was due to absence of cross-reacting antibody or insufficient antigen on the blot.

The results obtained in cross-immunoprecipitation studies are shown in Figures 14 and 15. It was found that the bovine anti-RS virus serum did not immunoprecipitate any PVM polypeptides, (Figure 14). In contrast the murine anti-PVM serum but not pre-immune mouse serum did immunoprecipitate the nucleocapsid protein of RS virus, (Figure 15). This provides evidence that PVM and RS virus

Figure 15. Cross-reaction of anti-PVM serum with the RS virus nucleocapsid protein.

The figure shows the fluorograph of a gel of PVM (P), RS virus (R) and mock infected (M) cell samples prepared as described in the legend to Figure 8 immunoprecipitated with anti-PVM or mouse pre-immune mouse serum. PVM specific polypeptides are labelled on the left.



nucleocapsid polypeptides are related.

3.1.11 Summary

The results presented in this chapter show the pneumovirus polypeptides identified in infected cell lysates, polyethylene glycol precipitates of infected cell supernatants and in *in vitro* translation experiments using unfractionated RNA from infected cells. The RS virus polypeptides that have been described by others are the L, G, F0 (cleaved to give F1 and F2 polypeptides), N, P, M, M2, 14K, 11K and 9.5K polypeptides. The major RS virus polypeptides observed during the course of this project were G, F1, N, P, M, M2 (probably two forms of differing mobility), 26K and 25K glycosylated polypeptide, F2, a 16K polypeptide (observed only *in vitro* and consisting of three polypeptides of different charge), and the 14K polypeptide. The L polypeptide of RS virus was not detected in any of the experiments described in this section probably due to this being the least abundant polypeptide for the reasons described in the Introduction (Section 1.1.1). Four major glucosamine labelled polypeptides were observed corresponding to G, F1, a 25K polypeptide and F2. The G protein was only observed using [³H]-glucosamine labelling probably due to its low methionine content although since the use of [³H]-leucine label failed to reveal this polypeptide its abundance in the system used during this project was probably quite low. The 26K and/or 25K

polypeptides probably represented a fragment of F1 since a polypeptide of similar mobility was immunoprecipitated by a F specific monoclonal antibody. The F1, N, P, M, M2 and 14K polypeptides were identified in [³⁵S]-methionine labelled cell lysates or PEG precipitates of supernatants from infected cells using immunoprecipitation or two dimensional gels. The 11K and 9.5K polypeptides were not observed the reason for this not being clear since the mRNA coding for these polypeptides would be expected to be relatively abundant since they occur towards the 3' end of the viral genome which is the first part transcribed. A 16K polypeptide observed *in vitro* which appeared to consist of 3 spots of differing charge may have included these polypeptides if they showed aberrant mobility. The experiments involving immunoprecipitation could have failed to identify these polypeptides due to them not being recognized by the antiserum.

The PVM polypeptides identified were L, G1, G2, F1, 44K, 42K, N, 39K, 35K, M, 25K, 24K, 23K, 20K, 19K, 18K, 16K, and 12K. The viral specificity of all of these apart from the 23K polypeptide was confirmed by immunoprecipitation using murine anti-PVM serum. The G1, G2, 44K and 42K polypeptides were glycosylated. The unglycosylated polypeptides except the L protein, which was detected when immunoprecipitation was used, were observed in *in vitro* translation experiments using RNA obtained from PVM infected cells.

The data on RS virus polypeptides is compared with the

published data of Collins et al. (1984a) and Norrby et al. (1986) in Table 6 and summarised in Table 8. The PVM polypeptides identified are compared with those observed by Cash et al. (1979) in Table 7 and the results summarised in Table 9.

Table 6 Comparison of RS virus polypeptides with published observations

Published results			Present results	
RS virus	M_r ($\times 10^{-3}$)		RS virus	M_r ($\times 10^{-3}$)
protein	Subtype A	Subtype B	protein	
L	200	200	L	190
G ¹	84	84	G	86.5
F0 ²	70	70	-	-
F1	48	46-47	F1	44.8 ³
N	42	42	N	39.4
P	35	32	P	33.1 ³
M	26	26	M	26.2
			GP26	25.6
			GP25	24.7
M2	24	24	M2	24.5 + 23.4
23K	23	-	22K	22.4 ⁴
F2	18-20	18.5-20	F2	19.4
-	-	-	16K	16.0
14K	14	-	14K	13.6
11K	11	-	-	-
9.5K	9.5	-	-	-

- 1 Strain dependent but subtype independent variations in mobility have been reported
- 2 F0 was not observed although the dimer F1,2 was observed in experiments described in Section 3.3.
- 3 This is a subtype A strain but the calculated M_r s differ from those published due to the use of different marker proteins.
- 4 This polypeptide was probably a F1 fragment showing inter-strain variability.

**Table 7 Comparison of PVM polypeptides observed with the
published data**

Published results		Present results	
PVM	$M_r (X10^{-3})$	PVM	$M_r (X10^{-3})$
protein		protein	
I	200	L	191
II	79	G1	76.4
III	63	G2	62.0
-	-	48K ¹	48.0
-	-	F1 ²	45.1
		44K	43.5
		42K	42.3
IV	42	N	40.3
V	40	39K	39.0
VI	35	35K	35.4
VII	28	M	27.3
		25K	25.1
VIII ³	26	24K	23.9
-	-	23K	22.9
		20K	20.2
IX	17	19K	19.3
X	16	18K	18.1
-	-	16K	15.8
XI	13.5	12K	12.0
XII	12		

1 Only observed amongst polypeptides synthesized *in vitro*.

2 Only observed amongst polypeptides synthesized *in vivo*.

3 Two polypeptides were sometimes observed in this region of the gel.

Table 8 Polypeptides of RS virus

RS virus protein	M_r ($\times 10^{-3}$)	Polypeptide identified		Acidic or basic	Identified by immuno- precipitation
		<i>in vivo</i>	<i>in vitro</i>		
L	190	+	-		+
G	86.5	+	-		+
F0	62.9	+	-	basic	+
F1	44.8	+	-	basic	+
N	39.4	+	+	acidic	+
P	33.1	+	+	acidic	+
M	26.2	+	+	basic	+
GP26	25.6	+	-		+
GP25	24.7	+	-		+
M2	23.4+24.5	+	+	basic	+
F2	19.4	+	-		+
16K	16.0	-	+	both ¹	+
14K	13.6	+	-		+

1 Three spots were observed corresponding to this band on two dimensional gels, one of which was basic, one slightly acidic and one acidic.

Table 9 Polypeptides of PVM

PVM protein	M _r (X10 ⁻³)	Polypeptide		Acidic or basic by immuno-	Identified by immuno- precipitation
		identified			
		<i>in vivo</i>	<i>in vitro</i>		
L	191	+	+		+
G1	76.4	+	-		+
G2	62.0	+	-		+
48K	48.0	-	+	basic	+
F1	45.1	+	-	basic	+
44K	43.5	+	-		+
42K	42.5	+	-		+
N	40.3	+	+	acidic	+
39K	39.0	+	+	acidic	+
35K	35.4	+	+	acidic	+
M	27.3	+	+	basic	+
25K	25.1	+	+	acidic	+
24K	23.9	+	+	basic	+
23K	22.9	+	-		-
20K	20.2	+	+	acidic	+
19K	19.3	+	+	acidic	+
18K	18.1	+	+	acidic	+
17K	17.0	-	+		+
16K	15.8	+	+	acidic ¹	+
12K	12.0	+	+		+ ²

1 Two spots were observed on two dimensional gels corresponding to two slightly acidic polypeptides differing in charge.

2 This polypeptide was not immunoprecipitated when it was labelled *in vitro*.

3.2 Characterization of PVM-specific monoclonal antibodies

3.2.1 Introduction

To study single PVM polypeptides antibodies reacting specifically with single viral polypeptides of the virus were required. This would enable precursor-product relationships between polypeptides to be established and enable polypeptides with similar or identical mobilities to be studied independently. Ideally polyclonal antibodies raised against purified viral polypeptides would be used. However, due to the cell associated nature of most of the infectivity the virus could not be readily purified. In addition the simplest way of obtaining purified polypeptides for immunization purposes, excising bands from gels, was not suitable since several viral polypeptides had similar mobilities. The N and 39K polypeptides and the 20K and 19K polypeptides for example would be difficult to separate satisfactorily. These problems could be overcome by producing monoclonal antibodies. This approach enables highly specific antibodies to be obtained despite immunizing animals with crude antigen preparations. In addition, once cell lines were established, qualitatively identical antibody preparations could be reproducibly obtained. There were some disadvantages in that the antibodies would only recognize single epitopes, so that a monoclonal antibody recognizing a protein involved in neutralization of the virus by polyclonal sera for example, might fail to neutralize the virus. The ability of the antibodies to

recognize denatured polypeptides would also vary. In addition some properties of antibodies are subclass specific so that not all monoclonal antibodies would bind to *S.aureus* protein A for example.

Hybridoma cell lines secreting PVM-specific antibodies were produced and screened as described in Materials and Methods. Several immunization protocols were used, in Fusion Experiment No.23 two intraperitoneal injections of a PEG precipitate of the supernatant of PVM-infected Balb/c μ T₁₂ cells followed by an intravenous injection of PVM purified from the supernatant of infected BS-C-1 cells was used. In Fusion Experiments Nos. 20 and 22 two intraperitoneal injections of PVM-infected BS-C-1 cells followed by one intravenous injection of the same material, sonicated and pelleted at 10,000g for 30 minutes to remove cell debris, were used. In Fusion Experiments Nos. 24, 25 and 26 two intraperitoneal injections of a PEG precipitate from PVM-infected Balb/c cells followed by two intravenous inoculations of the same material was used. In Fusion Experiments Nos. 4, 6 and 7 two intraperitoneal injections of PVM-infected Balb/c cells were used. In the Fusion Experiments not mentioned above three intraperitoneal injections of PVM infected Balb/c cells were used. The sera of the mice showed an anti-PVM ELISA titre of at least 5000 in each case before the final boost which was given 4 days before removal of the spleens and fusion of the cells with myeloma cells. The pooled hyperimmune sera of these mice had a neutralization titre of 45,000 and recognized most of the

virus specific polypeptides in immunoprecipitation experiments as previously described (3.1.4). Pre-immune sera from these mice showed neutralization titres of less than 100 and failed to immunoprecipitate virus specific polypeptides. The numbers of hybridoma cell lines, the numbers of these producing PVM-specific antibodies and the characterization of these antibodies are described in this Section.

3.2.2 Hybridoma cell lines obtained

A total of 26 Fusion Experiments were performed, 22 using mice immunized with PVM infected Balb/c cells and 4 using mice immunized with partially purified virus, as described above. The numbers of clones grown up and the numbers of these producing PVM-specific antibody are shown in Table 10. Fusion Experiments in which no hybrids grew up are not included for clarity. The reason for the low numbers of hybrids obtained in most experiments is not clear. There are three possible explanations for this, firstly there may have been deficiencies in technique, secondly the reagents used in the process may have varied with respect to fusion efficiency and thirdly the state of the cells may have been important. The final three Fusion Experiments were performed on the same day with no noticeable variation in technique but the results varied considerably. In Fusion Experiment No.26 Ag8.653 cells were used rather than NS-1 cells, although these cells had also been used in earlier Fusions Experiments (numbers 11 and 19) where no improved efficiency

Table 10 Details of fusions performed

Number and date of fusion	Antigen used for final boost	Myeloma cells used	Number of colonies obtained	Number of clones producing PVM-specific antibodies
1, 14/ 6/85	a	NS-1	19	2
2, 15/ 6/85	a	NS-1	5	1
3, 19/ 6/85	a	NS-1	21	3
4, 8/ 7/85	a	NS-1	3	0
5, 9/ 7/85	a	NS-1	1	0
8, 23/ 7/85	a	NS-1	17	2
9, 6/ 8/85	a	NS-1	44	0
11, 13/ 8/85	a	Ag8.653	13	2
12, 16/ 8/85	a	NS-1	6	0
13, 19/ 8/85	a	NS-1	2	0
14, 23/ 8/85	a	NS-1	1	0
17, 28/ 9/85	a	NS-1	15	3
19, 3/10/85	a	Ag8.653	41	6
24, 29/11/85	b	NS-1	35	23
25, 29/11/85	b	NS-1	11	2
26, 29/11/85	b	Ag8.653	371	182

1. a PVM-infected Balb/c cells inoculated intraperitoneally.

b Partially purified PVM from BS-C-1 cells inoculated by the intravenous route.

was observed. In Fusion Experiments Nos. 25 and 26 commercially produced polyethylene glycol solution was used, and this again did not produce consistent results. Changing to a different batch of medium and obtaining another vial of NS-1 cells (from a laboratory where fusions were successfully performed), similarly did not affect the results. The reason for the unsatisfactory results of most fusions was not therefore determined. The only satisfactory result was obtained using commercial polyethylene glycol solution and Ag8.653 as opposed to NS-1 cells.

The Fusion Experiments from which hybridoma cell lines were finally obtained are indicated in Table 11. The number of clones grown up indicates the number of cell lines that were successfully transferred from 96 well plates to 12 well tissue culture clusters. The number of clones characterized indicates the number of cell lines that were cloned 3 times, grown up in mice to produce ascites fluid and the antibodies characterized by the tests described below. After the initial screening the results of the ELISA assays during the screening of cloned cells for antibody production frequently failed to give clear positive results. A possible explanation for this is given in the following section. This meant that recloned cells could not be selected on the basis of antibody production and probably accounts for the large proportion of cell lines that did not give virus specific antibody reactions in the tests described.

**Table 11 Numbers of viable hybridoma cells producing
PVM-specific antibody**

Fusion number	Number of clones grown up	Number of clones characterized
19	6	2
24	21	4
26	38	19

3.2.3 Immunofluorescence using monoclonal antibodies

Some variability was observed in the results of ELISA assays, perhaps due to variation in the fixation of the cells allowing different degrees of access of the monoclonal antibodies to intracellular antigens. Indirect immunofluorescence using acetone fixed cells was used to establish the viral specificity of the antibodies more conclusively. Screening was carried out against PVM infected, RS virus infected or uninfected BS-C-1 cells. Six monoclonal antibodies gave positive results by this method of screening. The results are shown in Table 12 and representative photographs of the appearances of the fluorescently labelled cells are presented in Figure 16. The absence of fluorescence observed with monoclonal antibody 26/1/A4, (Figure 16, 7 and 8), was typical of the results observed with monoclonal antibodies listed as giving negative reactions in Table 12. The PVM-specific antibodies recognized antigens expressed internally in infected cells. These antigens were present in localized regions of the cell either in a perinuclear location or associated with the virus-specific filamentous processes. These processes, first described by Faulkner *et al.* (1976) have also been detected by Pringle and Parry (1980) using scanning electron microscopy to detect *Staphylococcus aureus* bound to virus specific antibody bound to viral antigen on the cell surface. These filaments are a distinctive feature of *Pneumovirus* infected cells not being observed in cells infected with other viruses, Parry *et al.*

Table 12 Results of immunofluorescence tests

Immunofluorescence observed with:-				Antibodies showing this pattern of fluorescence
PVM infected:-		uninfected:-		
fixed cells	unfixed cells	fixed cells	unfixed cells	
+	+	-	-	19/1/C9
+	-	-	-	26/1/A1
				26/1/A2
				26/1/D7
				26/1/E11
				26/4/C4 ¹
-	-	-	-	19/2/E11
				24/3/A7
				24/3/H8
				24/5/A1
				26/1/A1
				26/1/A4
				26/1/B4
				26/1/B10
				26/1/B11
				26/1/C4
				26/1/C7
				26/1/D7
				26/1/E3
				26/1/H8
				26/3/F10

Immunofluorescence observed with:-				Antibodies showing this
PVM infected:-		uninfected:-		pattern of fluorescence
fixed	unfixed	fixed	unfixed	
cells	cells	cells	cells	

26/3/H5

26/2/E1

26/4/B3

26/4/B7

+

-

+

-

24/1/A1

26/5/E2

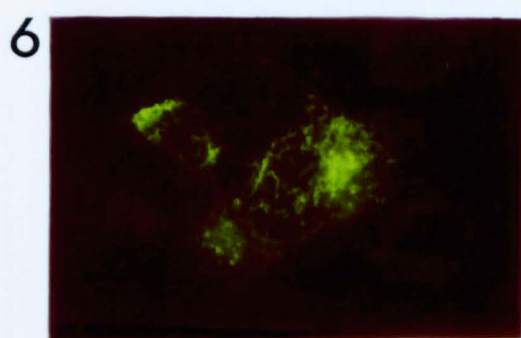
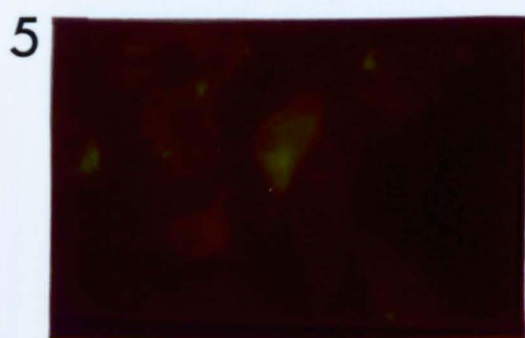
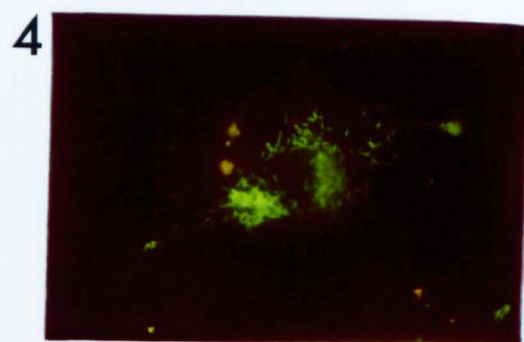
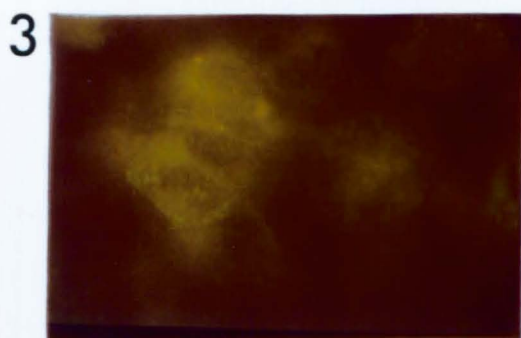
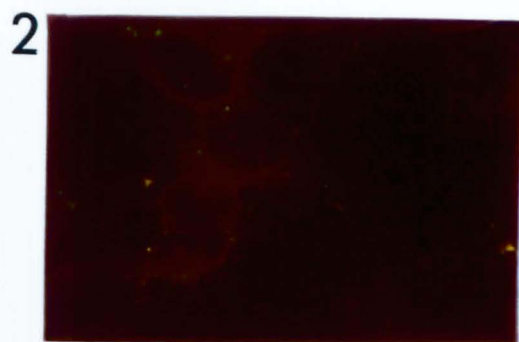
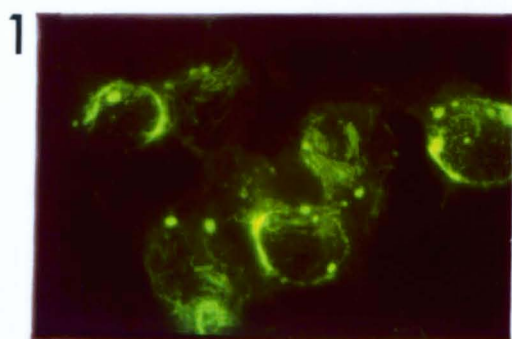
1 This antibody also reacted with fixed RS virus infected cells

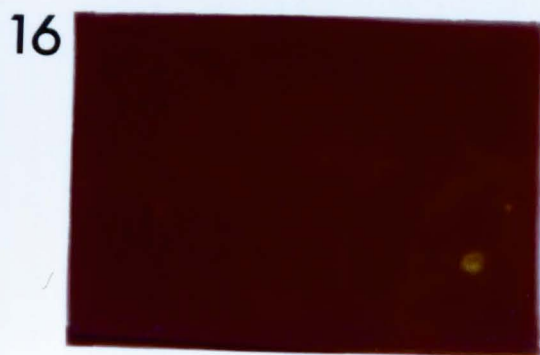
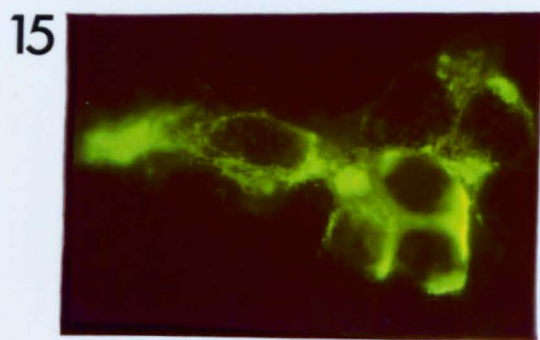
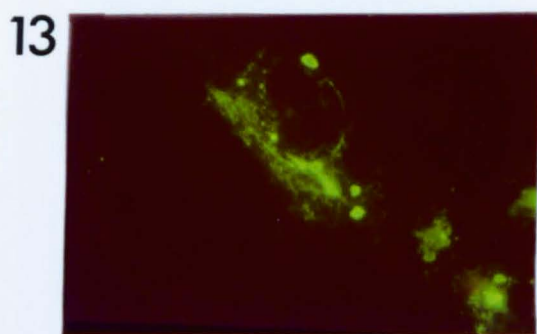
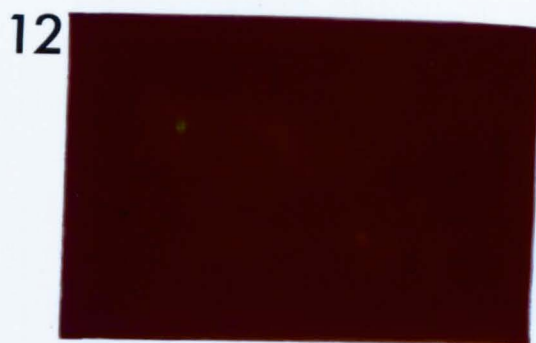
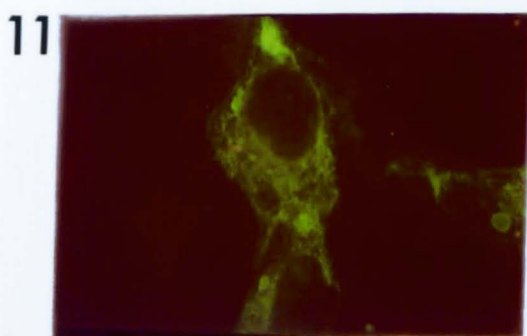
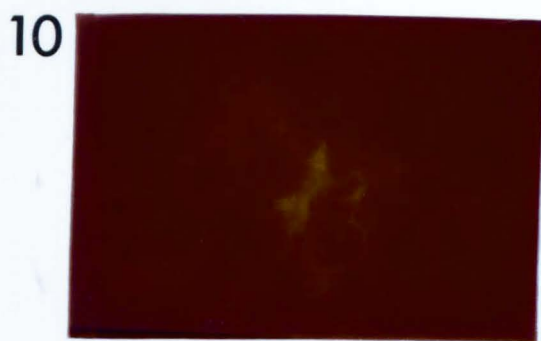
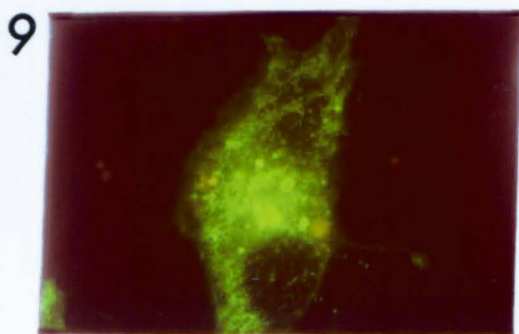
**Figure 16 Immunofluorescence reaction of monoclonal
antibodies with acetone fixed, PVM or RS virus infected
BS-C-1 cells**

PVM and RS virus infected BS-C-1 cells grown on glass coverslips or multiwell slides as described in Materials and Methods were fixed 24 hours after infection with acetone and incubated successively with monoclonal antibody and fluorescein conjugated anti-mouse immunoglobulins as described in Materials and Methods (Section 2.5.21). The slides were examined at a magnification of 1000X as described in Section 2.5.21.

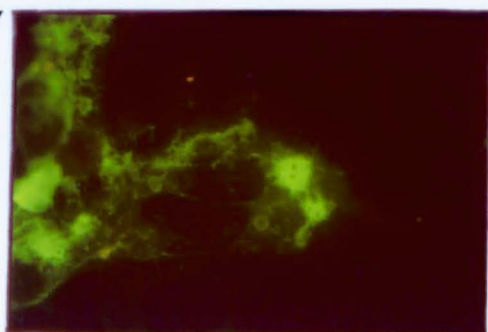
Odd numbered figures show staining with PVM infected cells and even numbered figures show staining with RS virus infected cells. Number 23 and 24 show staining of antibodies 26/4/C4 and 26/5/E2 with uninfected BS-C-1 cells. The antibodies used are indicated below.

- 1,2 anti-PVM serum
- 3,4 43.1 H8 (anti-RS virus F protein mAb)
- 5,6 111.2a (anti-RS virus G protein mAb)
- 7,8 26/1/A4
- 9,10 19/1/C9
- 11,12 26/1/A1
- 13,14 26/1/A2
- 15,16 26/1/D7
- 17,18 26/1/E11
- 19,20 26/4/C4
- 21,22 26/5/E2





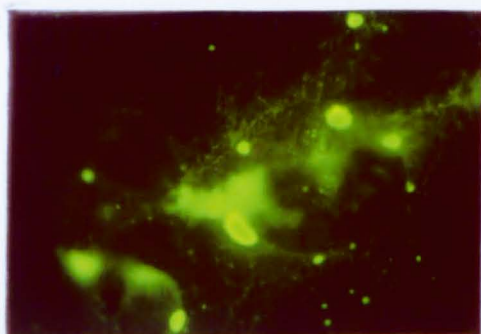
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18



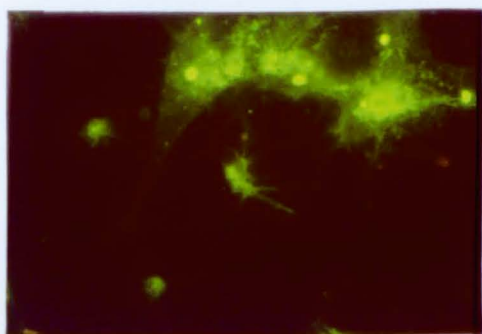
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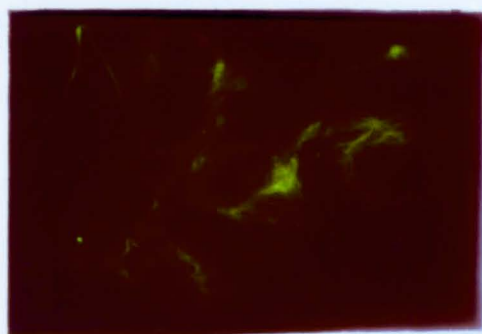
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22



23



24

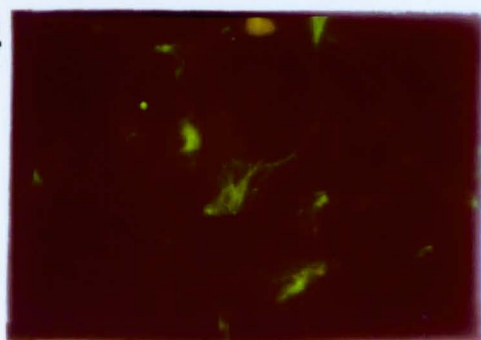


Figure 17 Immunofluorescence reaction of monoclonal
antibodies with unfixed PVM and RS virus infected BS-C-1
cells

Immunofluorescence was carried out essentially as described in the legend to Figure 16 except that the cells were not fixed. Odd numbered figures show the reaction with PVM infected cells and even numbered figures show the reaction with RS virus infected cells.

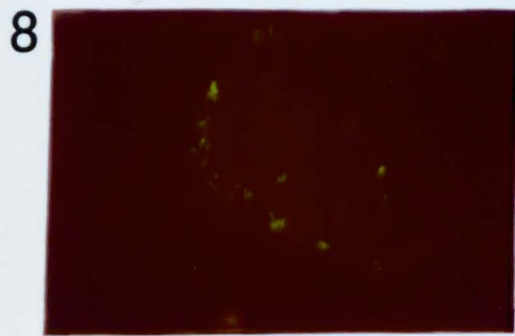
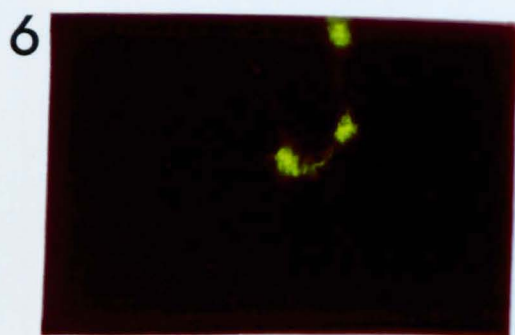
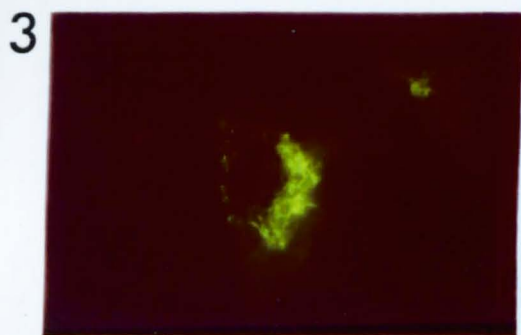
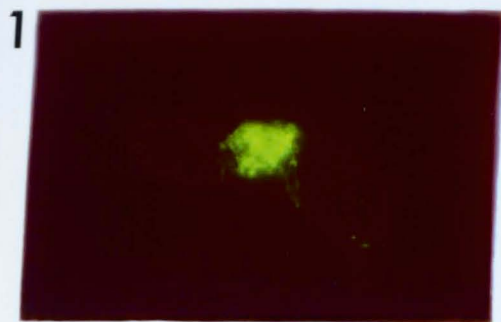
1,2 anti-PVM serum

3,4 19/1/C9

5,6 43.1 H8 (anti-RS virus F protein mAb)

7,8 111.2a (anti-RS virus G protein mAb)

9 26/1/A1



(1979).

Monoclonal antibody 26/4/C4 was observed to cross-react with RS virus infected cells (Figure 16, 19 and 20) but the other antibodies did not show evidence of cross-reaction. This reaction did not appear to be due to reaction with a cellular protein because it did not give a positive reaction with uninfected cells (Figure 16, 23). Antibody 26/5/E2 gave a positive reaction with mock infected cells (Figure 16, 24) as well as with PVM and RS virus infected cells (Figure 16, 21 and 22) and therefore appeared to recognize a cellular antigen, or to react with a viral and a cellular antigen as has been described by Srinivasappa et al (1986). The pattern of fluorescence given by this antibody with PVM infected cells was different from that obtained with RS virus or mock infected cells. This suggests that if the antibody recognizes a cellular protein its distribution is altered in infected cells.

The monoclonal antibodies were also screened against unfixed cells and the results of some of these studies are shown in Figure 17. It was found that only antibody 19/1/C9 showed a positive reaction with unfixed cells suggesting that this antibody and not the other antibodies reacted with an antigen expressed on the surface of infected cells (Figure 17, 3). Cell surface fluorescence was also observed when RS virus infected cells were stained with anti-F and anti-G monoclonal antibodies, (Figure 17, 6 and 8). Figure 17, 9 shows the absence of fluorescence observed using monoclonal antibody 26/1/A1 as a representative negative

result.

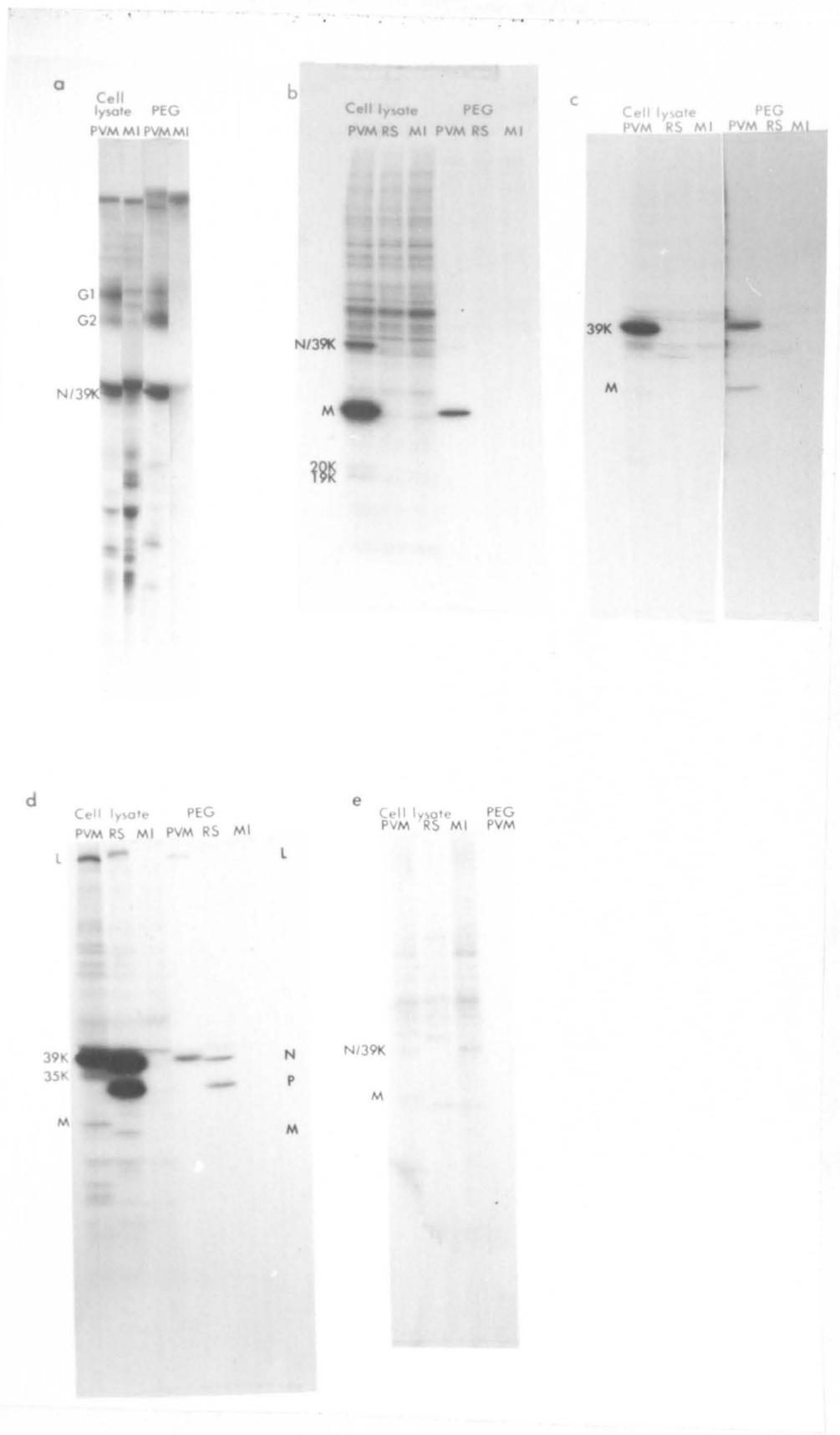
3.2.4 Immunoprecipitation with monoclonal antibodies

Immunoprecipitation of radiolabelled lysates and of PEG precipitates of the supernatants of PVM, RS virus and mock infected cells was performed to identify the polypeptides recognized by the monoclonal antibodies. It was found that 3 of the 25 monoclonal antibodies tested precipitated [^{35}S]-methionine labelled polypeptides, the results being shown in Figure 18. Antibody 19/1/C9 precipitated both polypeptide G1 and polypeptide G2, (Figure 18, a) antibody 26/1/E11 reacted with the M polypeptide of PVM, (Figure 18, b), antibody 26/4/C4 precipitated the N or 39K polypeptide, (Figure 18, c). Figure 18, e shows the negative result that was obtained with antibody 26/1/A1 which is representative of the negative results obtained with all the other antibodies. In another experiment a different result was obtained with antibody 26/4/C4 (Figure 18, d). This antibody was found to precipitate the L polypeptide in addition to the N or 39K polypeptide from PVM infected cells and also to precipitate polypeptides L, N and P from RS virus infected cells. The results obtained in Western blots described below showed that the antibody recognized the N or 39K polypeptide in PVM-infected cells and P in RS virus infected cells. The co-precipitation of the RS virus N protein by monoclonal antibodies specific for the P protein has been observed previously (Gimenez et al. (1984)). The co-precipitation of the L and N or 39K

**Figure 18 Immunoprecipitation with anti-PVM monoclonal
antibodies**

Cell lysates and PEG precipitates of the supernatants (PEG) of [³⁵S]-methionine labelled cells infected with PVM, RS virus (RS) or uninfected (MI) were immunoprecipitated with monoclonal antibodies and analysed by SDS-PAGE using 6-15 per cent resolving gels. The antibodies used are indicated below.

- a) 19/1/C9
- b) 26/1/E11
- c) 26/4/C4
- d) 26/4/C4 performed after observing the cross
reaction with RS virus shown in Figure 16.
- e) 26/1/A1



polypeptides and also the co-precipitation of the 3 RS virus proteins was probably due to the association of these polypeptides in nucleocapsid structures. The association of a polypeptide with a high M_r with nucleocapsid structures has been observed for the rhabdovirus, vesicular stomatitis virus (Bishop & Roy, 1972; Emerson & Wagner, 1972; Szilagyi & Uryayev, 1973), measles virus (Stallcup et al., 1979; Robbins et al., 1981), the paramyxoviruses Newcastle disease virus (Hamaguchi et al., 1983; Colonno & Stone 1976) and SV5 (Buetti & Choppin, 1977) as well as for RS virus (Peeples & Levine, 1979) and PVM (Cash et al., 1979a). It was subsequently established that the monoclonal antibody 26/4/C4 reacted with the 39K polypeptide (Section 3.4.6).

The cross reaction between the two glycoproteins observed using monoclonal antibody 19/1/C9 indicates a possible relationship between these two polypeptides and this is discussed later (Section 3.3). Other polypeptides were irregularly immunoprecipitated by this antibody (e.g. the N protein in Figure 18, a) and were considered to be precipitated non-specifically.

Only certain subclasses of antibodies bind to *Staphylococcus aureus* protein A so some of the antibodies might have failed to immunoprecipitate antigens due to failure to bind protein A. To overcome this problem formalin fixed *S. aureus* was incubated overnight at 4°C in a 1 in 10 dilution of rabbit anti-mouse immunoglobulin and used to adsorb antibody-antigen complexes from antibody-radiolabelled

sample mixtures for 3 hours on ice. This procedure did not result in the identification of the antigenic specificity of any further antibodies.

3.2.5 Western blotting with anti-PVM monoclonal antibodies

Western blots of cell lysates and PEG precipitates of the supernatants of uninfected, PVM and RS virus infected cells were probed with each of the monoclonal antibodies. Radioimmunoprecipitation enables antibodies to be characterized that do not recognize denatured proteins. Western blots provide a less ambiguous means of identifying the specificity of the antibodies since the problem of co-precipitation observed with radioimmunoprecipitation does not occur. Two of the antibodies recognized the PVM N or 39K proteins, 26/1/A2 and 26/4/C4 (Figure 19, b and c). Figure 19, a shows the absence of a reaction with antibody 26/1/A1 which is representative of the the results obtained with antibodies giving a negative result. The failure of 26/1/A2 to recognize the N or 39K protein in cell lysates may have been due to the relatively low abundance of viral proteins in cell lysates on the blot compared with that in the PEG precipitate sample. It can be seen that the cross reaction of 26/4/C4 with RS virus was due to a cross reaction with the RS virus phosphoprotein.

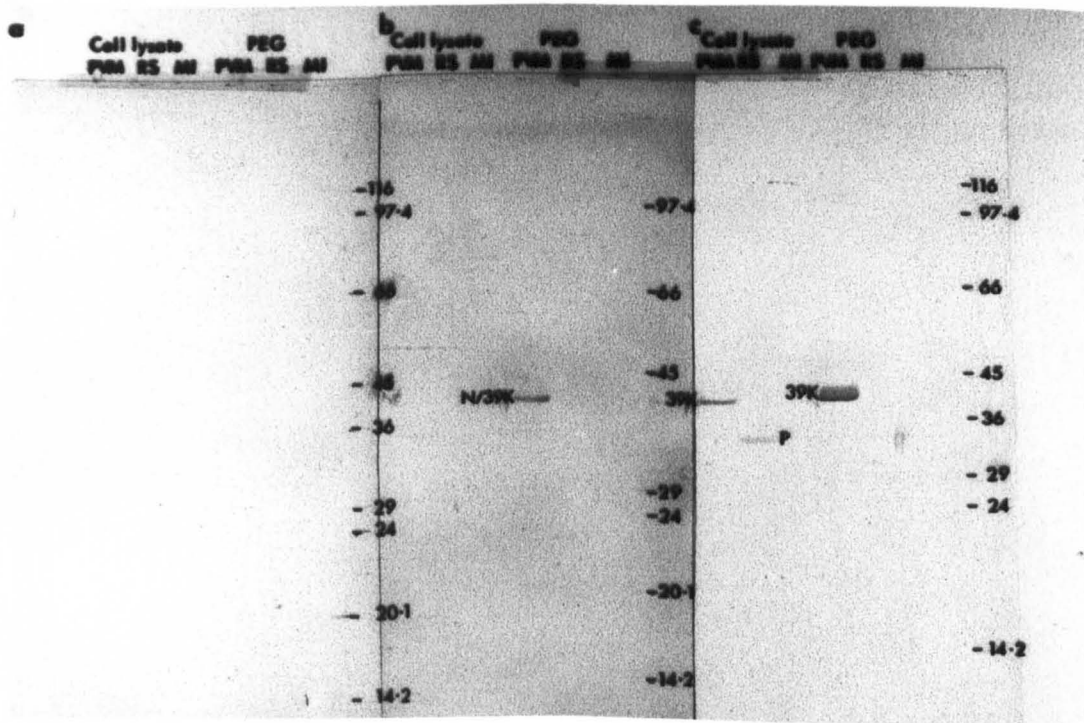
3.2.6 Neutralization and haemagglutination inhibition

The supernatants of the two surviving clones from Fusion Experiment No.19 and those of all the colonies

Figure 19 Reaction of monoclonal antibodies in Western blots.

Polypeptides from cell lysates and PEG precipitate samples were separated by SDS-PAGE and transferred to nitrocellulose. The blots were cut into strips and reacted with antibodies as described in Section 2.5.19. The antibodies used in each case are indicated below.

- a) 26/1/A1
- b) 26/1/A2
- c) 26/4/C4



producing PVM-specific antibody obtained in Fusions Experiments Nos.24, 25 and 26 were screened for neutralizing and haemagglutination inhibiting activity. Only antibody 19/1/C9 showed haemagglutination inhibiting activity,. Ascitic fluid containing antibody from this cell line had haemagglutination inhibition titres of 20,480 compared to titres of less than 20 for all other ascitic fluids tested.

Neutralizing activity was observed in the supernatants of 20 clones from Fusion Experiment No.24 and 2 clones from Fusion Experiment No.26. None of these clones could be examined further however since they either failed to recover from storage in liquid nitrogen or their supernatants no longer possessed neutralizing activity after recovery of the cells.

3.2.7 Summary

The results presented in this section show that of 6 monoclonal antibodies shown to be PVM-specific by immunofluorescence, the polypeptides recognized by 4 of them could be identified. The 19 monoclonal antibodies that gave negative or variable reactions in immunofluorescence experiments did not react in immunoprecipitation, Western blots, neutralization or haemagglutination inhibition assays. One of the monoclonal antibodies, 26/4/C4, recognised the 39K protein, one, 26/1/A2, recognized the N or 39K protein, one, 26/1/E11 recognized the matrix protein and one, 19/1/C9, recognized a virus glycoprotein. The latter monoclonal antibody inhibited haemagglutination of

murine erythrocytes by PVM to high titre implying that the polypeptide that it recognizes may be the haemagglutinin of the virus. PVM is unique amongst the pneumoviruses in possessing haemagglutination activity. None of the surviving hybridoma produced antibodies neutralized infectivity of the virus in BS-C-1 cells. Neutralizing activity was associated with the antibody produced by 20 clones that did not survive storage in liquid nitrogen. With the exception of antibody 26/4/C4 none of the monoclonal antibodies cross-reacted with RS virus antigens. This anti-39K protein antibody immunoprecipitated the nucleocapsid protein and the phosphoprotein of RS virus. A summary of the properties of the PVM specific monoclonal antibodies is shown in Table 13. These antibodies provided specific reagents for studying processing of individual viral polypeptides in PVM infected cells.

Table 13 Reactions of PVM specific monoclonal antibodies¹

Antibody	IF with		RIP ²	WB ²	HI	neutralization
	fixed	unfixed			titre	titre
	cells	cells				
19/1/C9	+	+	G1+G2	-	20,480	<10
26/1/A1	+	-	-	-	<20	<10
26/1/A2	+	-	-	N or 39K	<20	<10
26/1/D7	+	-	-	-	- ³	- ³
26/1/E11	+	-	M	-	<20	<10
26/4/C4	+	-	39K, L (P, N, L)	39K, (P)	<20	<10

1 Abbreviations used are as follows, IF-immunofluorescence,
RIP-radioimmunoprecipitation, - indicates no reaction

WB -Western blotting, - indicates no reaction

HI -haemagglutination inhibition with 4 HA units of PVM and
murine erythrocytes

2 RS virus polypeptides which cross react are shown in
brackets

3 Supernatants gave negative results, no ascites was
available

3.3 GLYCOPROTEINS OF PNEUMONIA VIRUS OF MICE

3.3.1 Introduction

The envelope glycoproteins of RS virus have been extensively studied both as proteins (Fernie *et al.* 1985; Gruber & Levine 1983, 1985a & b; Hendricks *et al.* 1987; Lambert & Pons 1984; Routledge *et al.* 1986; Samson *et al.* 1986; Tsutsumi *et al.* 1987; Walsh & Hruska 1983; Walsh *et al.* 1984a, 1985, 1986) and as antigens (Bangham *et al.* 1986; Elango *et al.* 1986; Johnson *et al.* 1987b; Olmsted *et al.* 1986; Pemberton *et al.* 1987; Stott *et al.* 1986; Walsh *et al.* 1984b, 1987a&b; Ward *et al.* 1983; Wertz *et al.* 1987). As was discussed in Section 1.1.2 these studies showed that the G glycoprotein has a peptide backbone with a M_r of 36,000 which is processed in two stages, first a rapid stage probably due at least in part to N-linked glycosylation giving a polypeptide with a M_r of 45,000 followed by a slower step leading to the mature glycoprotein with a M_r of 84,000-90,000. In addition two different glycosylated bands with M_r s of 45,000 and 50,000 related to G have been observed the significance of which is not clear (Routledge *et al.* 1986). The F glycoprotein has been shown to be involved in fusion of infected cells to form syncytia and is synthesized as a 70K polypeptide which is then cleaved to give disulphide bonded polypeptides of about 50K and 20K.

The glycoproteins of PVM are significantly different from those of RS virus (Cash *et al.* 1979a and Section 3.1.5). The main aim of the experiments described in this section was to study the PVM glycoproteins and their

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synthesis in more detail. RS virus was used in parallel with PVM in most of the experiments to serve as a control for the techniques employed. The RS virus G protein could not be satisfactorily radiolabelled with [³⁵S]-methionine, 4,5-[³H]-leucine or [¹⁴C]-protein hydrolysate, so PVM glycoproteins could only be compared with the RS virus F glycoprotein in experiments using these radiolabelled amino acids. The experiments described in this section describe the glycoproteins of PVM, their relationship to each other and the nature of the linkages between the carbohydrate and protein.

3.3.2 Partial proteolysis of glycoproteins G1 and G2

Since the glycoproteins G1 and G2 were both immunoprecipitated by monoclonal antibody 19/1/C9 it was necessary to establish whether this was due to the two polypeptides being related, or if they were simply co-precipitated due to an association between them. In an attempt to resolve this question the G1 and G2 bands were excised from gels of PVM polypeptides, immunoprecipitated with either polyclonal anti-PVM serum or with monoclonal antibody 19/1/C9, and subjected to partial digestion with proteases. The two glycoprotein bands were found to be highly resistant to digestion with the enzymes used, (*Staphylococcus aureus* V8 protease, chymotrypsin, trypsin and endoprotease Lys-C). No digestion was observed with the latter two enzymes when digestion was carried out according to the method of Cleveland et al. (1977) for 30 minutes

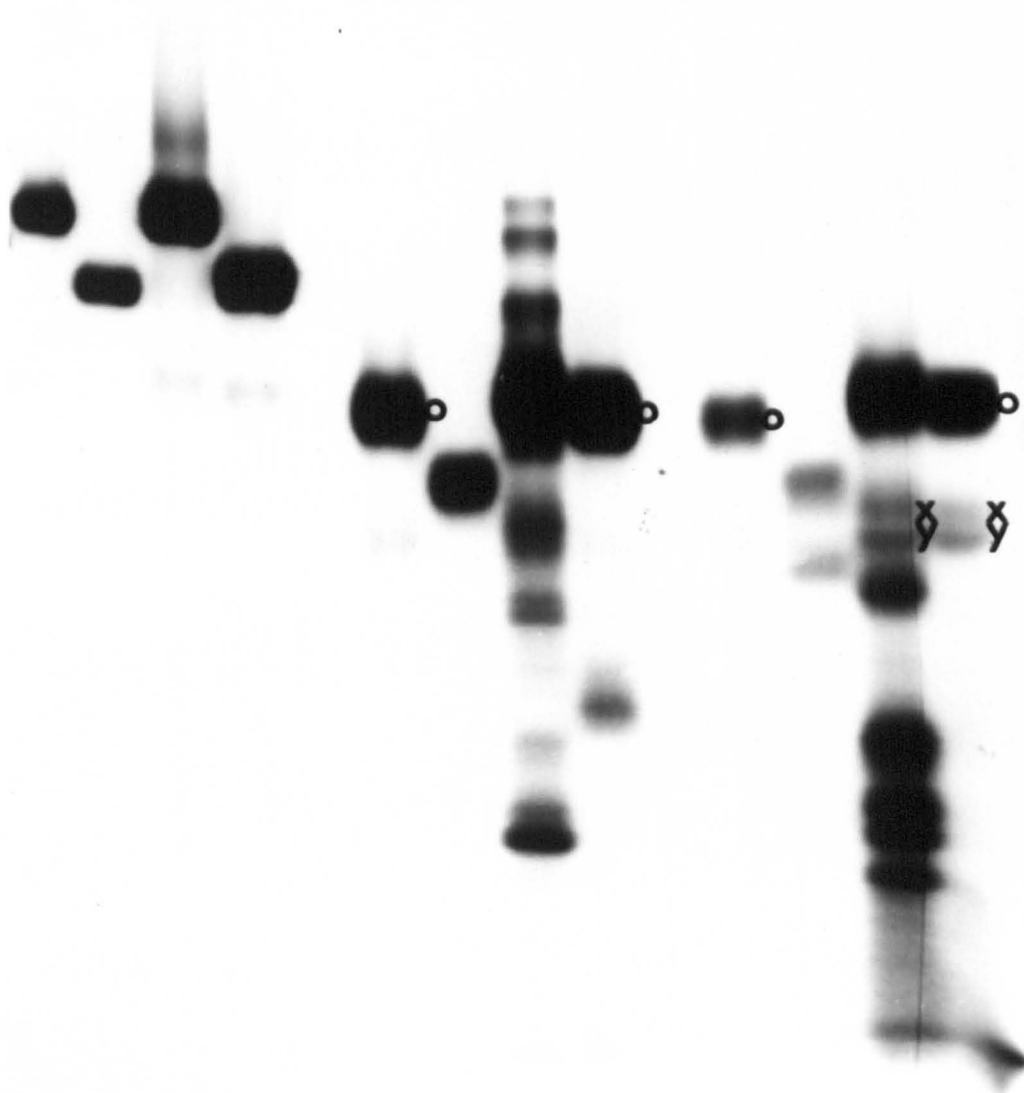
Figure 20 Partial proteolysis of PVM glycoproteins using
S.aureus V8 protease

The G1 and G2 polypeptides of PVM along with a polypeptide observed in tunicamycin treated, PVM infected cells were partially digested with *S.aureus* V8 protease prior to analysis by SDS-PAGE using a 6-15 per cent gradient gel as described in Section 2.5.22. The polypeptide bands were excised from gels of immunoprecipitates of [³⁵S]-methionine labelled PVM infected cells (labelled cell lysate in the figure) and PEG precipitates of the supernatants of such cells (labelled PEG) and digested with the concentration of *S.aureus* V8 protease indicated for 30 minutes. The G1t polypeptide was immunoprecipitated from lysates of tunicamycin treated, PVM infected cells labelled with [³⁵S]-methionine. The figure shows a fluorograph of the gel on which the digests were run. The circles indicate peptides common to G1 and G2.

Undigested
Cell lysate PEG
G1 G1t G1 G2

10 μ g V8 protease ml⁻¹
Cell lysate PEG
G1 G1t G1 G2

100 μ g V8 protease ml⁻¹
Cell lysate PEG
G1 G1t G1 G2



using each enzyme at 100µg per ml. Results of digestion with V8 protease at concentrations of 10 and 100µg per ml of the G1 and G2 glycoproteins isolated by immunoprecipitation with the monoclonal antibody are shown in Figure 20. One major band (o) was observed in all the samples which showed only slight mobility differences. These differences were not significant since they were no greater between G1 and G2 than between G1 from the cell lysate track and G1 from the PEG precipitate. The significance of the other bands observed in the G1 tracks is not clear since this polypeptide was more heavily labelled than the others. The difference in labelling intensity could effect the number of bands visible or there may be contaminating labelled polypeptides in the G1 band. Digests of G1 and G2 derived from the PEG precipitate sample shared two bands (x and y) which had similar mobilities. In order to establish whether the single strong band obtained was the same in each case the band was excised and redigested with chymotrypsin. No digestion of this band occurred so no definite conclusions could be drawn from these results.

The G1t band digested on these gels represented a band immunoprecipitated from cell lysates of PVM infected cells incubated in the presence of the N-linked glycosylation inhibitor tunicamycin. G1t had a similar mobility to G2, which was not well labelled in cell lysates in this experiment, and G1 was absent when cells were labelled in the presence of tunicamycin. This suggested that G1t was a form of G1 lacking N-linked sugars. The single band obtained

on digestion of G1t had a higher mobility than the band from the glycoproteins suggesting that it may have been an equivalent band without N-linked sugars. Further studies on the products immunoprecipitated from tunicamycin treated cells with antibody 19/1/C9 are presented later in this section.

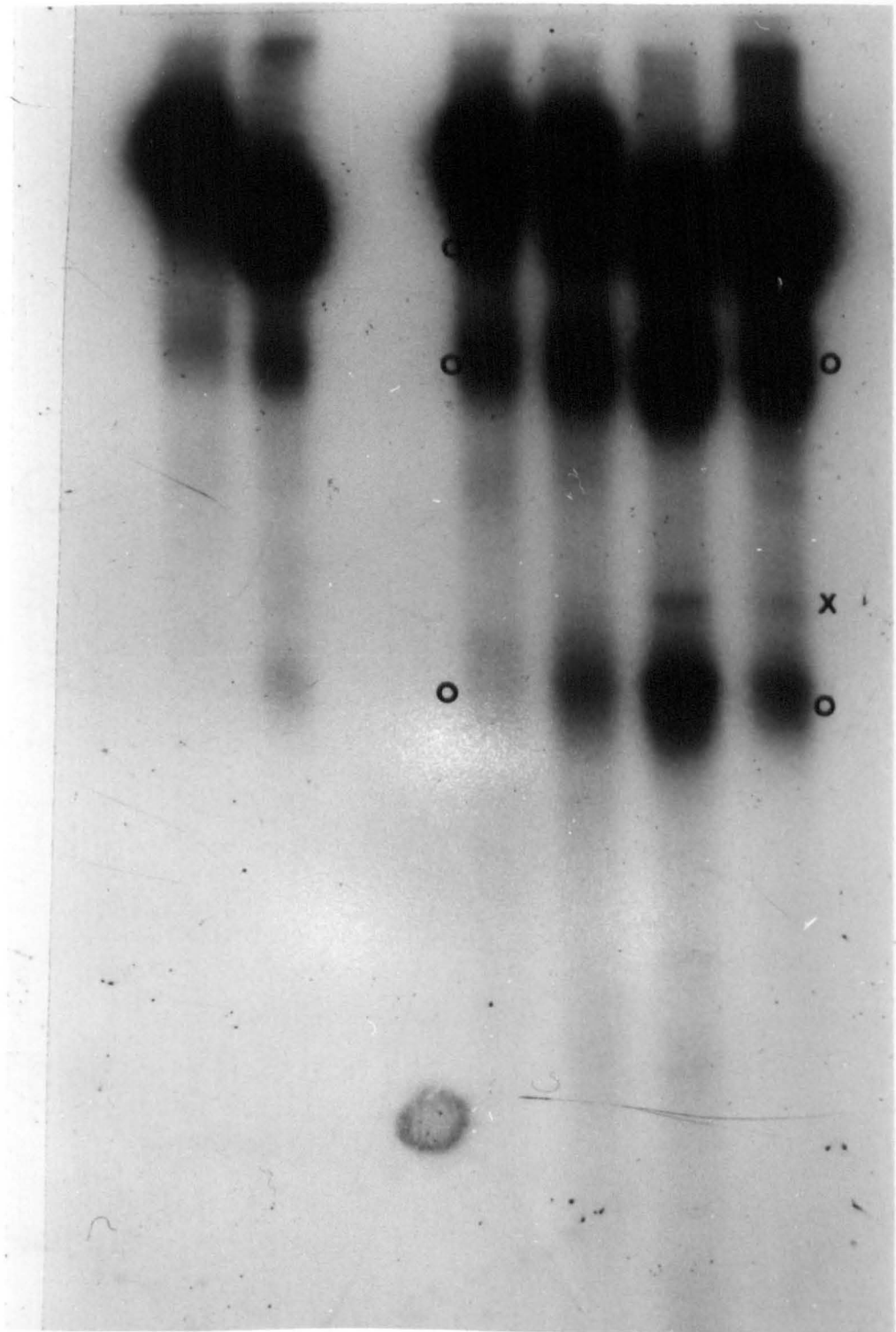
Figure 21 shows the results obtained on digestion of G1 and G2 with 100 or 1000 μ g per ml of chymotrypsin. The bands in Figure 21 were excised from a gel of samples immunoprecipitated with anti-PVM serum. Identical results were obtained when a similar digestion was carried out using bands excised from a gel of samples immunoprecipitated with monoclonal antibody 19/1/C9. The pattern of polypeptides observed was similar regardless of the source of the bands. Three digestion products were observed from G1 and three from G2. Two of the digestion products of G2 had similar mobilities to two products obtained on digestion of G1 (indicated by circles in the figure). The band obtained on digestion of G1 absent from digests of G2 (indicated in the figure by a circle adjacent to the digests of G1 but not G2) was of a similar size to G2 and therefore could not be compared with its digestion products. The band obtained on digestion of G2 but not G1 (labelled x in the figure) was relatively weak. A similar band was obtained on digestion of the G1t polypeptide (which had a similar mobility to G2) observed when infected cells were labelled in the presence of tunicamycin. This band may therefore represent a breakdown product of a contaminating host cell protein in

**Figure 21 Digestion of G1 and G2 polypeptides of PVM with
Chymotrypsin**

The G1 and G2 polypeptides of PVM were digested with chymotrypsin and the peptides resolved by SDS-PAGE using 15 per cent resolving gels. The G related polypeptides were excised from gels of [^{35}S]-methionine labelled PVM polypeptides immunoprecipitated from PEG precipitates of the supernatants of infected cells. The immunoprecipitation was carried out with the anti-PVM serum. The excised gel slices were inserted into the wells of the gel on which the peptides were to be resolved. The polypeptides were digested with chymotrypsin at the concentration indicated, (0, 100 or 1000 μg per ml), during migration through the stacking gel as described in materials and methods (Section 2.5.22).

G1 G2
0 0

G1 G2
100 1000 1000 100



this region of the gel. This band could also indicate that G2 and the G1t polypeptide shared a peptide of similar mobility. Since the two major digestion products of G1 and G2 obtained using chymotrypsin and the single major band obtained when V8 protease was used had similar mobilities regardless of which glycoprotein was digested the data are consistent with the two glycoproteins being related.

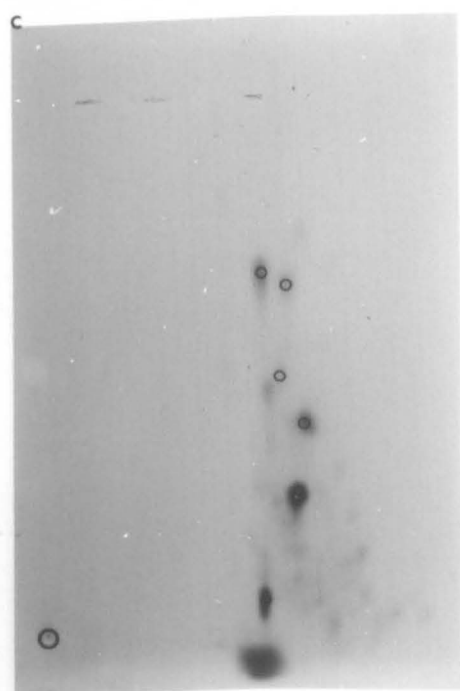
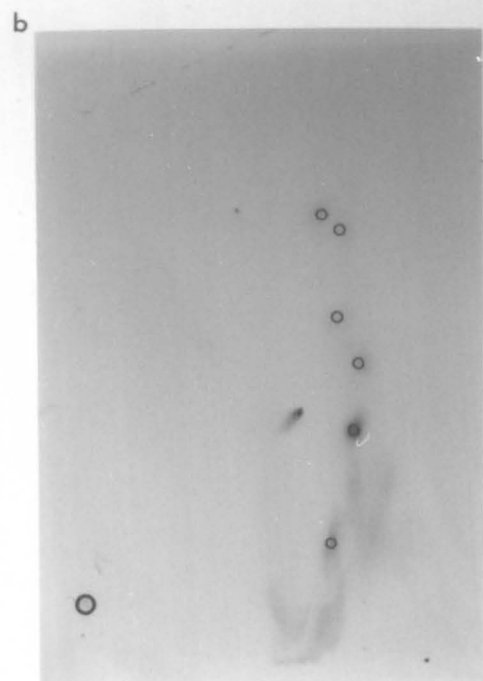
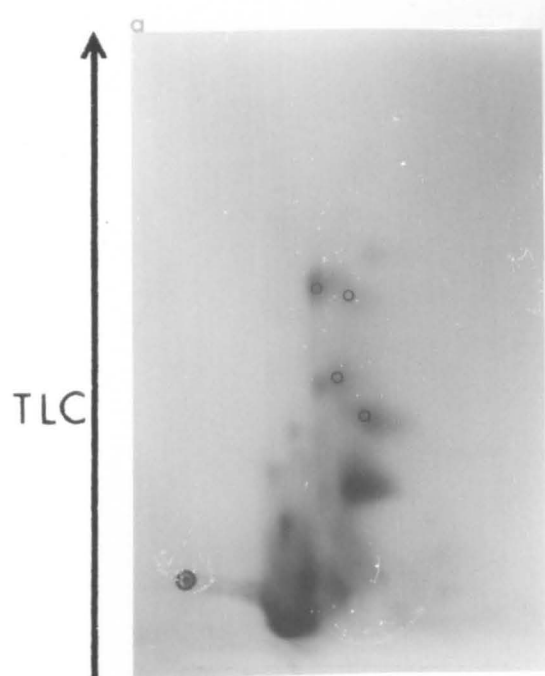
3.3.3 Tryptic peptide maps of G1 and G2

To try and establish the relationship between G1 and G2 more clearly the glycoproteins, excised from gels of [³⁵S]-methionine and [4,5-³H]-leucine labelled PVM polypeptides immunoprecipitated with anti-PVM mouse serum were digested with trypsin (50µg per ml for 23 hours) and analysed by 2-dimensional thin layer electrophoresis/chromatography followed by fluorography. Comparison of the peptide patterns obtained on digestion of G1 (Figure 22a), G2 (Figure 22b) and when the two digests were run together on the same TLC sheet (Figure 22c) show that the two polypeptides share many peptides (indicated by small circles) providing strong evidence that they are in fact related. Several unlabelled spots could not be identified as being present in both G1 and G2 digests due to insufficient label incorporation or insufficient resolution of the peptides. The large spot at the bottom of fluorographs a and c probably corresponded to glycopeptides which do not run in the second dimension (Montelaro *et al.* 1984; Salinovich, *et al.* 1986). The large circle at the

Figure 22 Tryptic peptide maps of G1 and G2

The G1 and G2 polypeptides were digested with trypsin and the peptides separated by two dimensional thin layer electrophoresis/thin layer chromatography as described in Section 2.5.24. The bands were excised from a gel of PVM polypeptides labelled with [^{35}S]-methionine (500 μCi per ml) and 4,5- [^3H]-leucine, (750 μCi per ml) and immunoprecipitated with anti-PVM serum. Spots common to all the fluorographs are indicated by small circles and the point of application of the samples indicated by large circles.

- (a) Fluorograph of the tryptic peptide map of G1.
- (b) Fluorograph of the tryptic peptide map of G2.
- (c) Fluorograph of the tryptic peptide map obtained on running digests of G1 and G2 together



- Thin-layer electrophoresis +

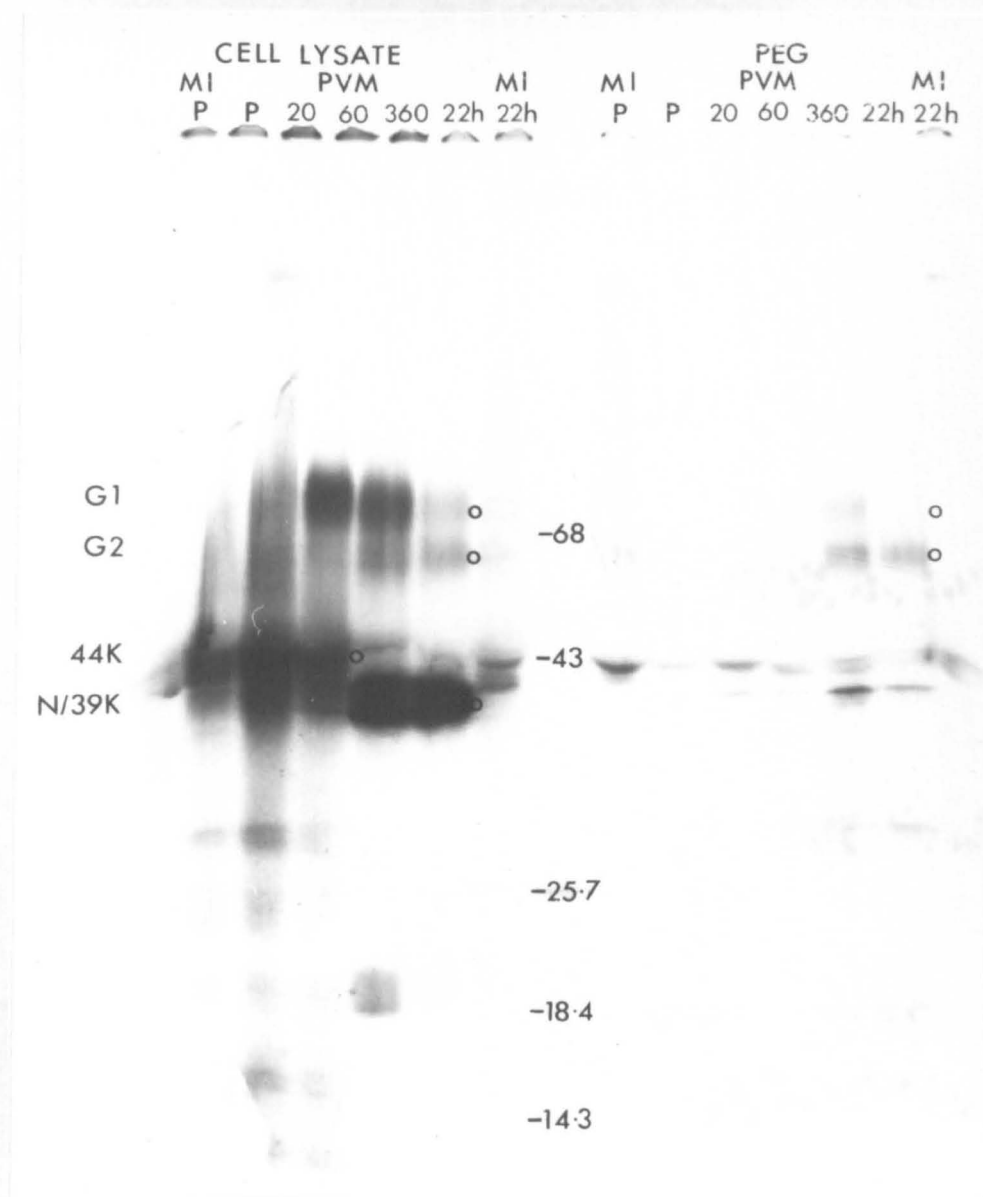
bottom left of each fluorograph indicates the point at which the sample was applied. The co-migration of spots in the mixed digest shown in part c indicates that the peptides were actually similar. The higher intensity of the spots in part a was due to a longer exposure of this fluorograph. A longer exposure of a peptide map similar to part b is not shown due to poor resolution of the peptides by electrophoresis.

3.3.4 Pulse chase studies of G1 and G2

In order to establish whether G1 and G2 represented different stages in the processing of a single glycoprotein pulse chase studies were performed. A 10 minute pulse label of [^{35}S]-methionine followed by various lengths of time in the presence of unlabelled methionine was used to follow the fate of methionine incorporated into polypeptides that could be immunoprecipitated by monoclonal antibody 19/1/C9. The result is shown in Figure 23. After the pulse period and after a pulse followed by a 20 minute chase period no PVM-specific polypeptides could be observed except possibly a 44K polypeptide and the N/39K polypeptide. After a 60 minute chase period G1 but not G2 could be detected in the cell lysate. G2 first became apparent after a 360 minute chase period when both polypeptides could also be observed to be present in culture supernatants. The amount of G2 relative to G1 that could be detected had increased after a 22 hour chase period. The higher mobility bands were not reproducibly observed and were considered to represent

**Figure 23 Characterization of G related polypeptides in
pulse chase experiments**

The polypeptides immunoprecipitated by the monoclonal antibody 19/1/C9 from PVM or mock infected cells labelled for 10 minutes with [^{35}S]-methionine (400 μCi per ml) or labelled in the same way and then chased for various times with unlabelled methionine are shown in this figure. The experiment was carried out as described in Section 2.5.13. The length of the chase period is indicated above each track in minutes except for the 22 hour (22h) samples (P indicates a pulse label only). Polypeptides immunoprecipitated from cell lysates are shown on the left (labelled cell lysate). Polypeptides immunoprecipitated from the PEG precipitates of supernatants of these cells are shown on the right (labelled PEG). Polypeptides immunoprecipitated from mock infected cell samples are indicated by MI above the track, all other tracks being polypeptides immunoprecipitated from PVM infected cell samples. The polypeptides immunoprecipitated from PVM infected cell samples are labelled on the left. Unlabelled bands were not considered to be virus specific due to the presence of similar polypeptides in the tracks of mock infected samples or irregular occurrence on gels. The positions of the molecular weight markers run in the centre track are indicated by their M_r s ($\times 10^{-3}$).



non-specifically precipitated polypeptides.

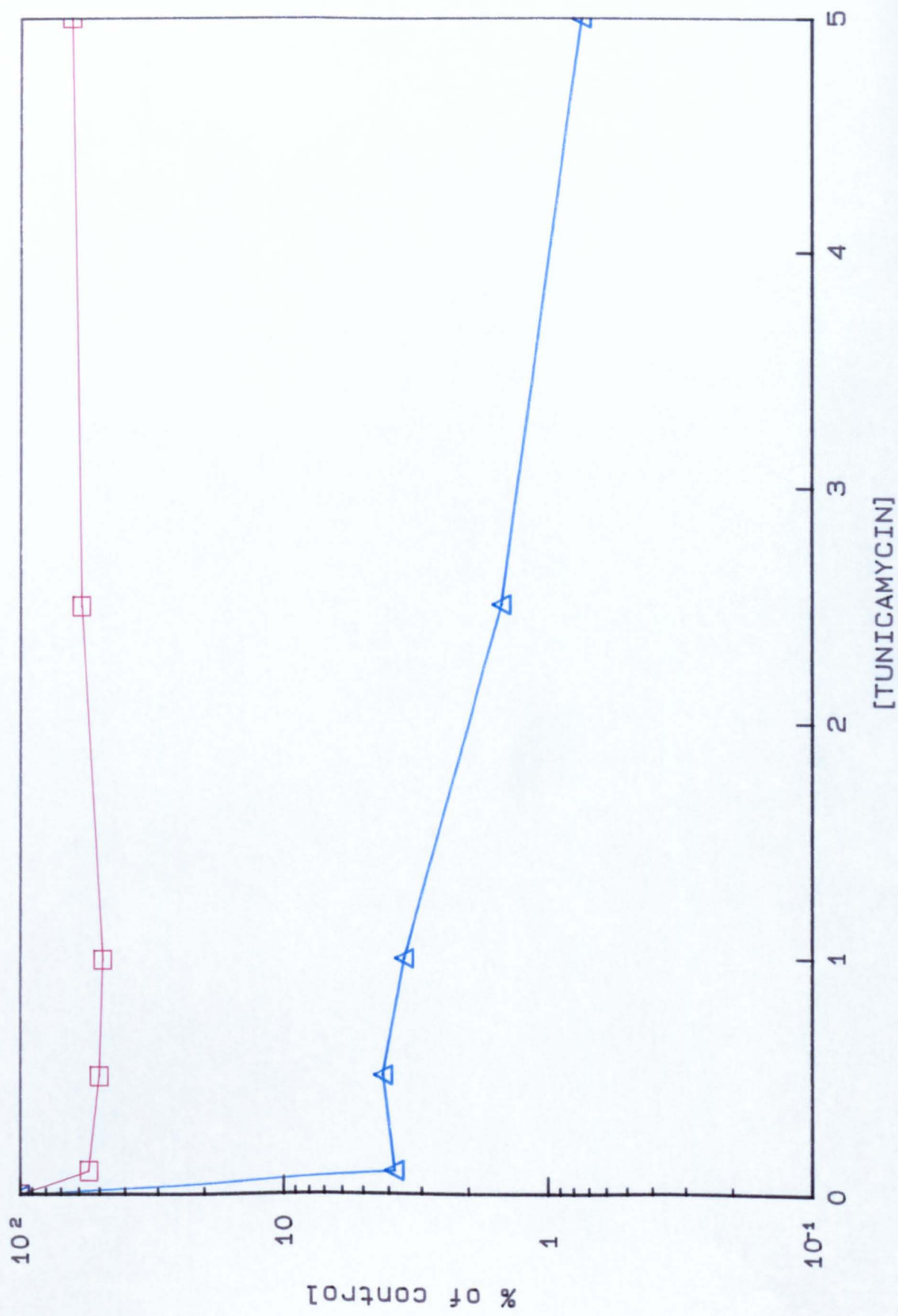
The apparent absence of a G related polypeptide following a short pulse or a pulse label followed by a short chase could have been due to insufficient labelling. If label incorporation increased during the chase periods sufficient label to observe immunoprecipitated glycoprotein might only be observed after longer chase periods. Alternatively the glycoprotein may have a precursor that is unstable or not recognized by the antibody. The former possibility appears unlikely since there was no overall increase in the background level of label incorporation. The 44K polypeptide probably represented a polypeptide non-specifically precipitated by the monoclonal antibody since several bands of high intensity were commonly observed in this region of the gel. Processing of G1 to give G2 was the most likely explanation for the decrease in intensity of the G1 band and increase in intensity of the G2 band after longer chase periods.

3.3.5 G related glycoproteins synthesized in the presence of an inhibitor of N-linked glycosylation

The two glycoproteins of RS virus show different patterns of glycosylation. The glycosylation of the RS F polypeptide appears to be entirely due to N-linked oligosaccharides which are not attached to the polypeptide chain in the presence of tunicamycin (Fernie et al. 1985; Gruber & Levine 1985a). The G polypeptide however, which is more highly glycosylated, shows only a slight increase in

Figure 24 Effect of different tunicamycin concentrations on incorporation of [^{35}S]-methionine into PVM infected cell polypeptides and on the virus yield from such cells.

PVM infected cells were incubated in the presence of various concentrations of tunicamycin (indicated in μg per ml). Some of the cells were scraped into suspension and assayed for infectivity 96 hours after infection. Similar infected cells were incubated from 48 to 72 hours post infection with [^{35}S]-methionine ($50\mu\text{Ci}$ per ml) in the presence of the same concentrations of tunicamycin. The cells were harvested, lysed and pre-cleared in the same way as when immunoprecipitation was to be performed (Section 2.5.15). Samples of the clarified lysate were then placed on filter paper discs and precipitated with 10 per cent TCA (three washes, the second one in boiling TCA solution). The discs were washed in ethanol:ether (1:1) and dried before measuring the radioactivity by scintillation counting. The figure shows a graph of infectivity (blue triangles) and label incorporation (red squares) against the tunicamycin concentration used. The infectivity and label incorporation values are expressed as a percentage of the values obtained when no inhibitor was used.



mobility when synthesized in the presence of tunicamycin indicating that most of the carbohydrate of the mature G protein is not N-linked. This inhibitor was therefore used to study the type of linkage of carbohydrate to peptide that occurred in the PVM glycoproteins.

Figure 24 shows the effect of various concentrations of tunicamycin on [^{35}S]-methionine incorporation and virus yield from PVM infected cells. The concentrations of tunicamycin selected for use in further experiments was $2.5\mu\text{g}$ per ml, which caused a significant reduction in virus yield (>99 per cent) whilst still allowing a reasonable level of label incorporation (>50 per cent of the control level). Figure 25 shows the effect of tunicamycin and monensin treatment (the effect of monensin is considered in the following section) of PVM infected cells on [^{35}S]-methionine labelled polypeptides immunoprecipitated with the monoclonal antibody 19/1/C9.

One band (G1t), could be identified in immunoprecipitates of lysates of tunicamycin treated PVM infected cells carried out using monoclonal antibody 19/1/C9. Two bands (G1t and G2t) were observed when PEG precipitates of the supernatants of tunicamycin treated PVM infected cells were immunoprecipitated. Since G1 was the predominant band immunoprecipitated from the cell lysate, whilst G1 and G2 had similar intensities when PEG precipitates were immunoprecipitated, it appeared likely that G1t represented G1 without its N-linked

**Figure 25 Effect of tunicamycin and monensin treatment of
PVM infected cells on the synthesis of G related
polypeptides**

PVM and mock infected cells were infected and labelled with [^{35}S]-methionine (50 μCi per ml), in the presence or absence of tunicamycin or monensin. The polypeptides were immunoprecipitated with monoclonal antibody 19/1/C9. The samples immunoprecipitated were either cell lysates (labelled cell lysate) or polyethylene glycol precipitates of the supernatants of the labelled cells (labelled PEG in the figure). The figure shows a fluorograph of the immunoprecipitated polypeptides analysed by SDS-PAGE using a 6-15 per cent gradient gel.

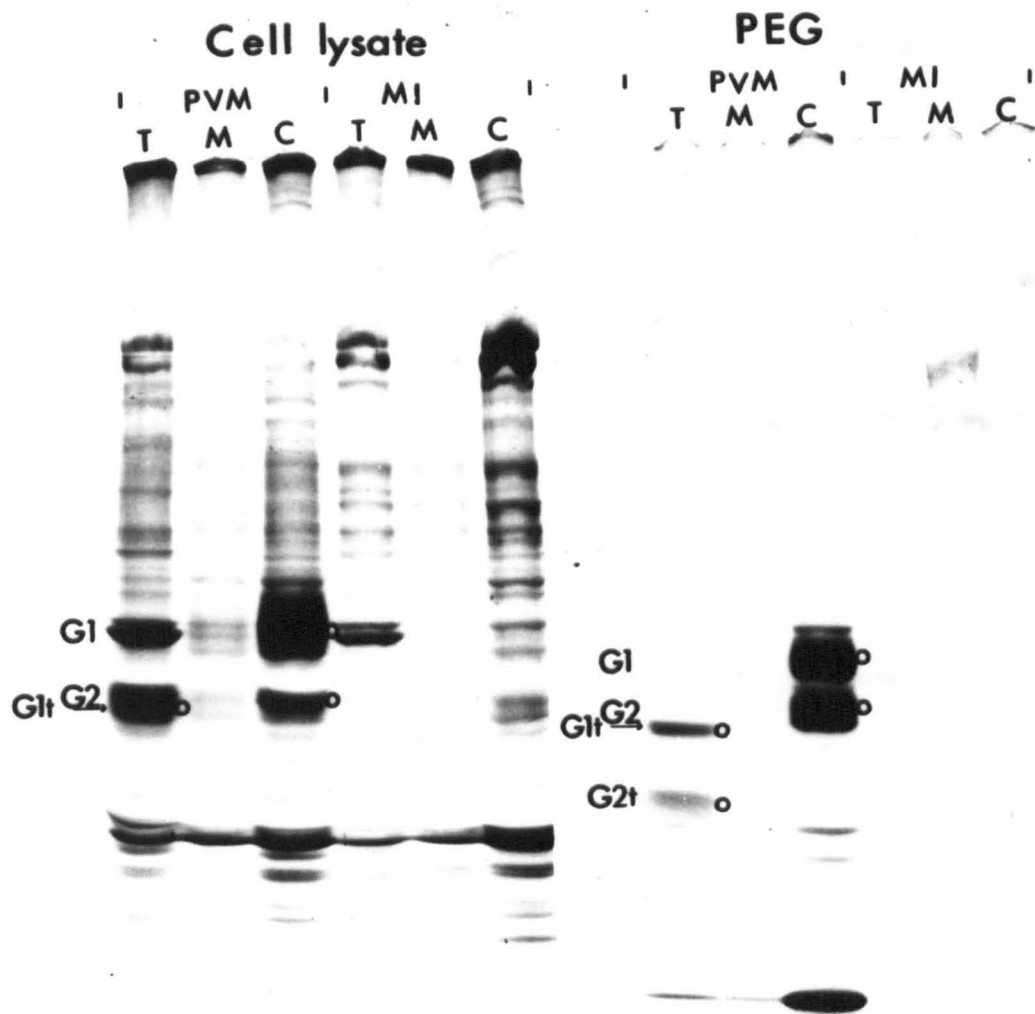
PVM- Samples from PVM infected cells.

MI- Samples from mock infected cells.

T- Samples from cells treated with tunicamycin
(2.5 μg per ml).

M- Samples from cells treated with monensin (0.8 μM).

C- Samples from untreated cells.



oligosaccharides and G2t represented G2 without its N-linked oligosaccharides.

3.3.6 G related glycoproteins synthesized in the presence of the ionophore, monensin

The monovalent ionophore monensin inhibits the passage of membrane and secretory proteins through the Golgi apparatus and this prevents the relatively late event of O-linked glycosylation (Johnson & Spear, 1983). This reagent has been used to inhibit glycosylation of the G protein of RS virus and results in a much greater increase in mobility than the use of tunicamycin (Fernie *et al.* 1985; Gruber & Levine 1985a). Such data have been used to suggest that most of the carbohydrate of G is O-linked.

Figure 26 shows the effect of different monensin concentrations on virus yield and [³⁵S]-methionine incorporation in PVM infected BS-C-1 cells. A concentration of 0.8μM was found to give a satisfactory combination of inhibition of virus yield and level of polypeptide synthesis. In the fluorograph shown in Figure 25 monensin treatment of infected cells was observed to cause a small increase of mobility in the RS F1 polypeptide as has been reported by Gruber & Levine (1985a), this may be due to inhibition of the later stages of N-linked carbohydrate processing. The RS G polypeptide could not be labelled with amino acids under the conditions used in this project so it was not possible to determine the effect of monensin on this polypeptide.

Figure 26 The effect of different monensin concentrations on [³⁵S]-methionine incorporation into PVM infected cells and the yield of virus from such cells.

PVM infected cells were incubated in the presence of various concentrations of monensin (indicated in μ M per ml). Some of the cells were scraped into suspension and assayed for infectivity 96 hours after infection. Similar infected cells were incubated from 48 to 72 hours post infection with [³⁵S]-methionine (50 μ Ci per ml) in the presence of the same concentrations of monensin. The cells were harvested, lysed and pre-cleared in the same way as when immunoprecipitation was to be performed (Section 2.5.15). Samples of the clarified lysate were then precipitated with TCA as described in the legend to Figure 24. The figure shows a graph of infectivity (blue triangles) and label incorporation (red squares) against the tunicamycin concentration used. The infectivity and label incorporation values are expressed as a percentage of the values obtained when no inhibitor was used.

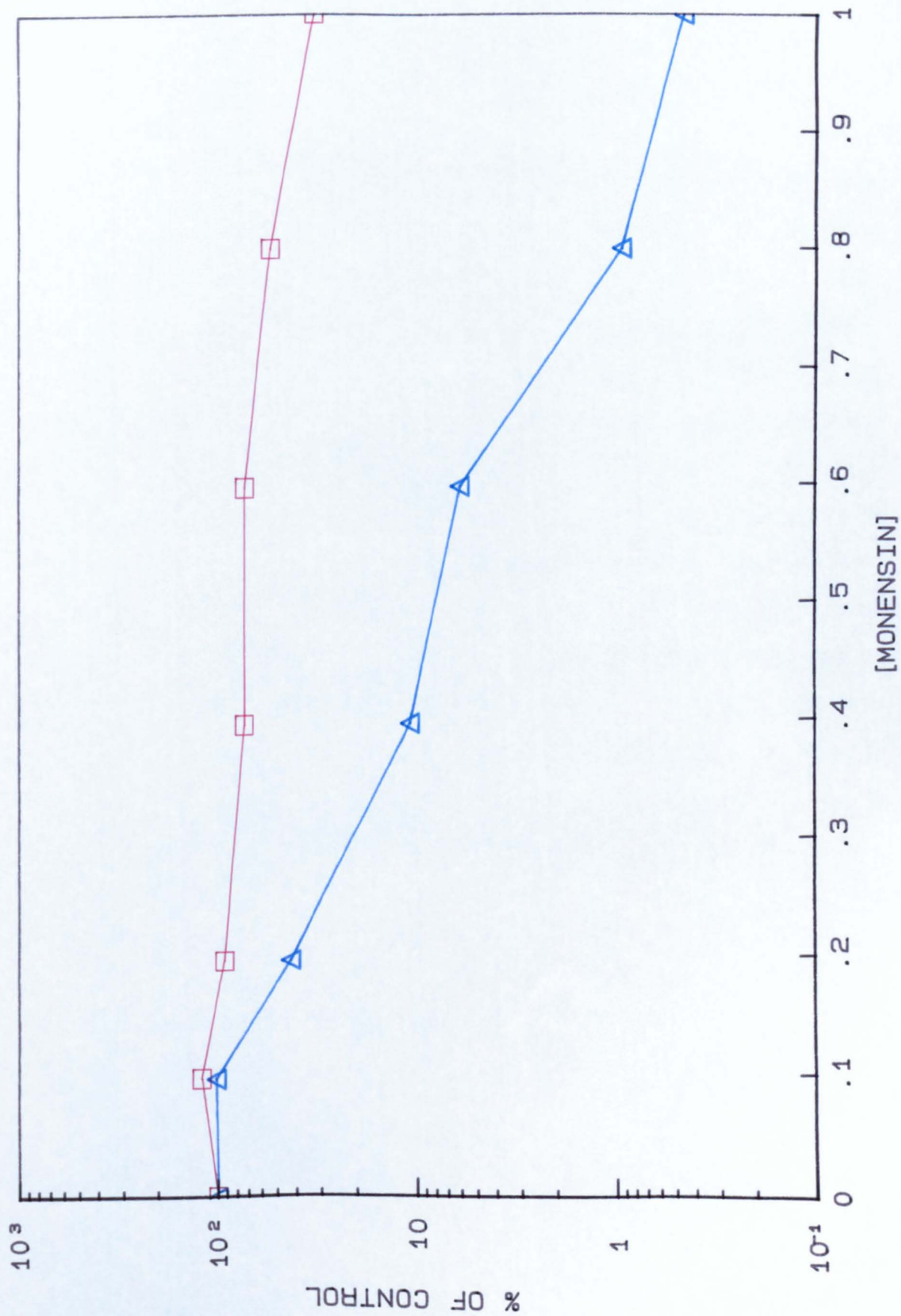
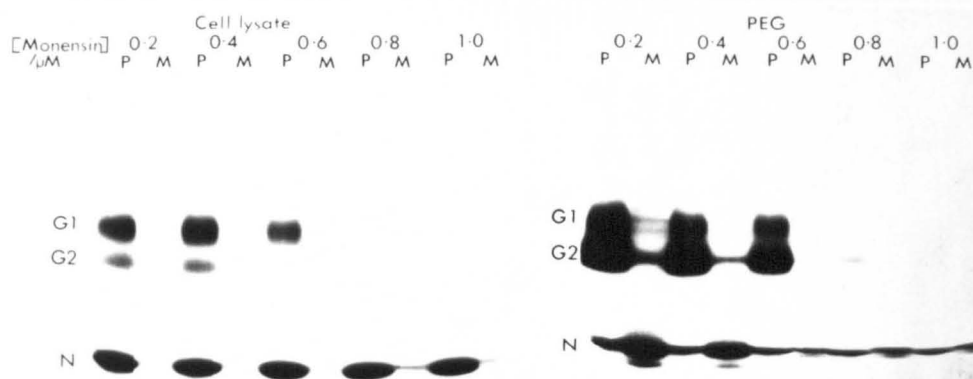


Figure 27 The effect of treatment of PVM infected cells with different monensin concentrations on the polypeptides immunoprecipitated by monoclonal antibody 19/1/C9.

PVM and mock infected cells were incubated for 48 hours and labelled for 24 hours with [^{35}S]-methionine (50 μCi per ml) in the presence of various concentrations of monensin (added immediately after the adsorption period). The figure shows fluorographs of two gels of immunoprecipitates of samples from such labelled cells. Monoclonal antibody 19/1/C9 was used to immunoprecipitate labelled cell lysates (labelled cell lysate in the figure) or PEG precipitates of the supernatants of cells (labelled PEG in the figure). The monensin concentration used during incubation of the infected cells and labelling is indicated (0.2, 0.4, 0.6, 0.8 or 1.0 μM). The PVM polypeptides immunoprecipitated by the monoclonal antibody are indicated on the left.

P- Immunoprecipitates of samples from PVM infected cells.

M- Immunoprecipitates of samples from mock infected cells.



**Figure 28 Absence of an effect of monensin on synthesis of
the 39K polypeptide of PVM**

PVM and mock infected cells (PVM and MI) were incubated for 48 hours in the presence of tunicamycin (2.5 μ g per ml, T) monensin (0.8 μ M, M) or were untreated (C) and were then labelled with [35 S]-methionine (50 μ Ci per ml) for 24 hours in the presence of the same inhibitors. The cells were lysed and immunoprecipitated with monoclonal antibody 26/4/C4. The figure shows a fluorograph of these immunoprecipitates analysed by SDS-PAGE using a 6-15 per cent gel.



No G related polypeptides could be observed in the samples from monensin treated PVM infected cells, the use of differing concentrations of monensin revealed either the usual pattern of polypeptides or no specifically precipitated polypeptides (Figure 27). The polypeptide precipitated in the cell lysate sample in this figure was found to be N (by partial digestion with chymotrypsin) rather than a G related polypeptide. The absence of any bands related to G1 or G2 in the samples from monensin treated PVM infected cells could have been due to the overall level of protein synthesis being reduced so that the poorly labelled G polypeptides could no longer be observed. Alternatively the failure to detect G related polypeptides in monensin treated cells could have been due to the presence of a form of G that was unstable or not recognized by the antibody. The reduction in label incorporation observed for the glycoprotein bands when infected cells were labelled in the presence of monensin was not observed for the 39K band when the samples were immunoprecipitated with the anti-39K monoclonal antibody 26/4/C4 (Figure 28). This observation argues against the failure to detect the G polypeptide in monensin treated PVM infected cells being due to a reduction in the overall level of polypeptide synthesis.

3.3.7 Effect of tunicamycin on [³H]-glucosamine labelled PVM and RS virus polypeptides.

Figure 29 and 30 show the effect of tunicamycin and

monensin on PVM and RS virus glycoproteins labelled with [³H]-glucosamine, immunoprecipitated from the cell lysates and PEG precipitates respectively. The effect of monensin is considered in the next section. The PVM samples were immunoprecipitated with anti-PVM serum whilst the RS virus samples were immunoprecipitated with anti-F (43.1) and anti-G (111.2a) monoclonal antibodies.

The F1 and F2 polypeptides of RS virus were present only at low levels in the supernatant and F0 was totally absent. A small amount of G was immunoprecipitated by the anti-F monoclonal antibody from the supernatant of RS virus infected cells (Figure 30). No sugar labelled polypeptides related to F could be detected in samples from tunicamycin treated, RS virus infected cells as was expected for a glycoprotein with only N-linked oligosaccharides. The G glycoprotein of RS virus showed only a slight increase in mobility when precipitated from cells incubated in the presence of tunicamycin. The G related bands in the 45K region of the gel were absent when samples were prepared from tunicamycin treated cells suggesting that this polypeptide had only N-linkages as would be expected of the precursor of G.

The 42K polypeptide in samples from PVM infected cells differed from the G related p45 polypeptide of RS and the RS F1 polypeptide in being relatively unaffected by the presence of tunicamycin during labelling. The 42K polypeptide in PVM infected cell lysates is more readily apparent in a longer exposure of the gel shown in Figure 29,

Figure 29 The effect of tunicamycin and monensin on
[³H]-glucosamine labelled PVM and RS virus polypeptides
occurring in the lysates of infected cells.

PVM, RS virus or mock infected cells were incubated for 48 hours at 33°C and then labelled for 24 hours using [³H]-glucosamine hydrochloride (100μCi per ml). Tunicamycin and monensin were used at 2.5μg per ml and 0.8μM, respectively. The PVM and mock infected cell lysates were immunoprecipitated with the anti-PVM serum whilst the RS virus cell lysates were immunoprecipitated successively with the anti-F monoclonal antibody (43.1) and the anti-G monoclonal antibody (111.2a). The figure shows a fluorograph of the immunoprecipitates analysed on a 6-15 per cent gradient gel. PVM specific polypeptides are labelled on the left and RS virus specific polypeptides on the right.

PVM- Samples immunoprecipitated from PVM infected cell lysates.

MI- Samples immunoprecipitated from mock infected cell lysates.

RS- Samples immunoprecipitated from RS virus infected cell lysates.

anti-PVM, 43.1 and 111.2a indicate the antibodies used for the immunoprecipitations.

T- Cells were incubated and labelled in the presence of tunicamycin

M- Cells were incubated and labelled in the presence of monensin

C- Cells were incubated and labelled in the absence of any inhibitors.

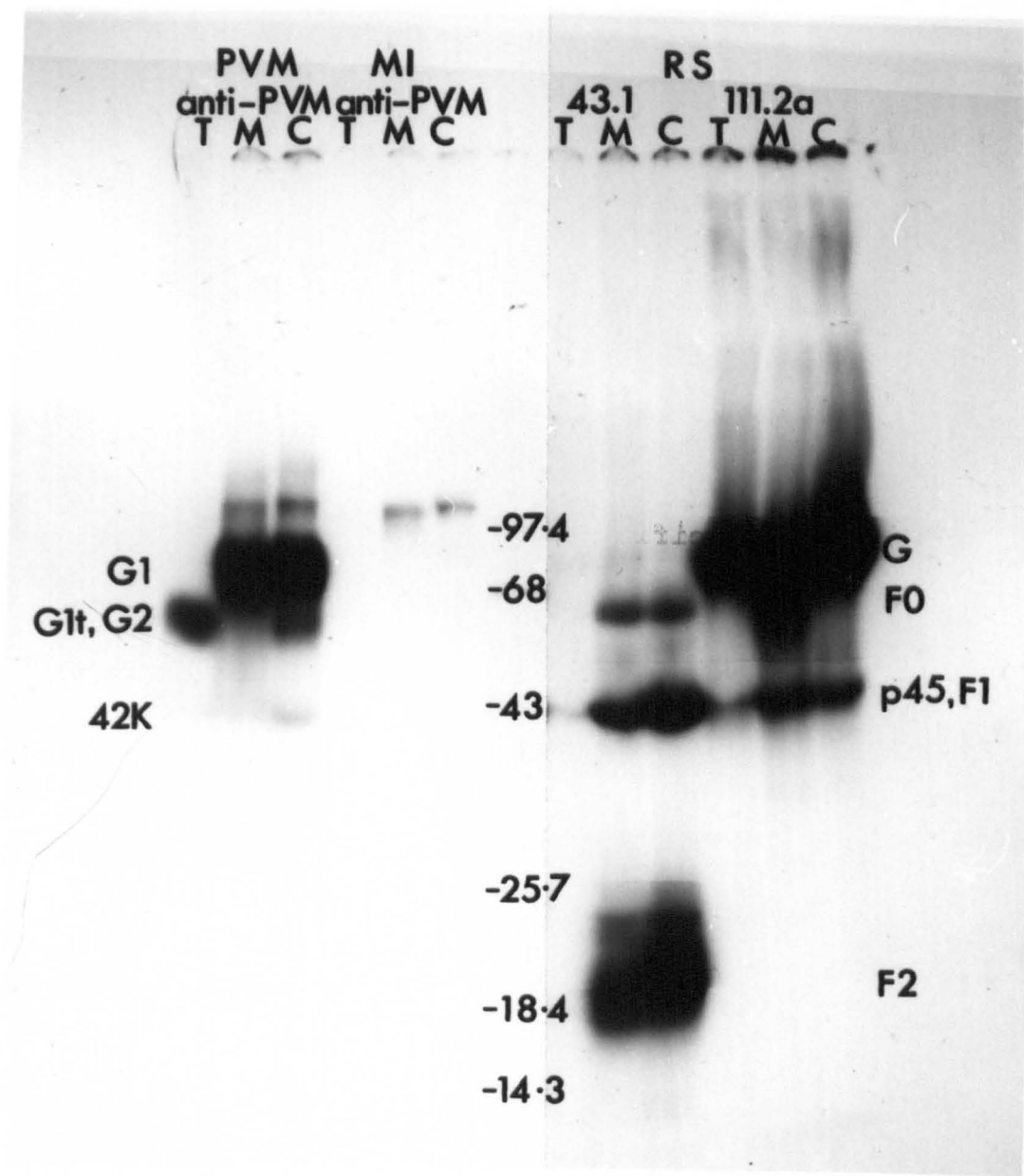


Figure 30 [³H]-glucosamine labelled virus polypeptides in
the PEG precipitates of tunicamycin and monensin treated
cells

Polyethylene glycol precipitates of the supernatants of the [³H]-glucosamine labelled cells described in the legend to Figure 29 were studied by immunoprecipitation. The figure shows a fluorograph of the labelled polypeptides immunoprecipitated with the antibodies indicated and analysed by SDS-PAGE on a 6-15 per cent gel. PVM specific polypeptides are labelled on the left and RS virus specific polypeptides on the right.

PVM- Polypeptides immunoprecipitated from the PEG precipitate of the supernatant of PVM infected cells

MI- Polypeptides immunoprecipitated from the PEG precipitate of the supernatant of mock infected cells.

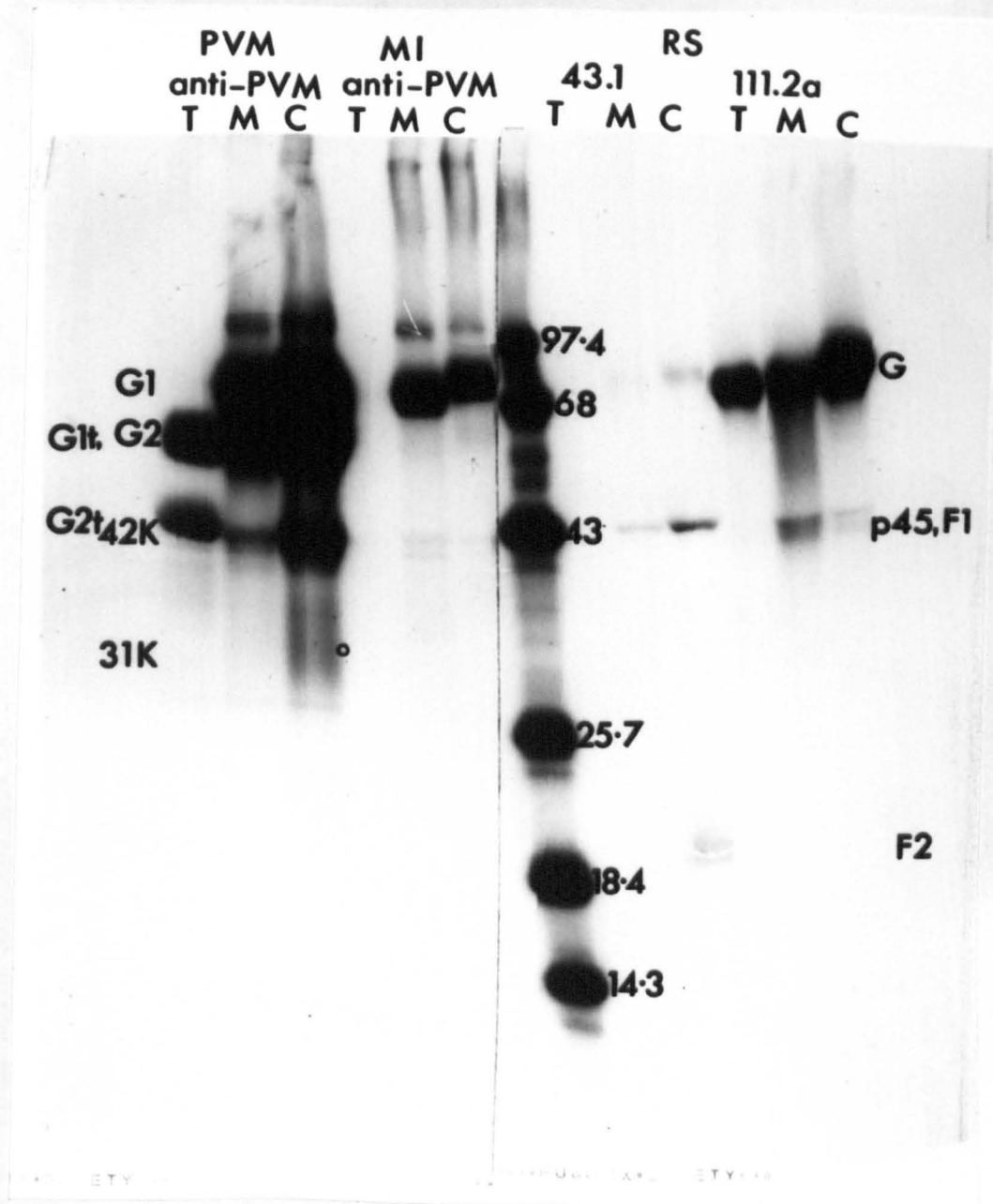
RS- Polypeptides immunoprecipitated from the PEG precipitate of the supernatant of RS virus infected cells

anti-PVM, 43.1 and 111.2a indicates the antibody used for the immunoprecipitation

T- The cells were incubated and labelled in the presence of tunicamycin (2.5µg per ml).

M- The cells were incubated and labelled in the presence of monensin, (0.8µM).

C- The cells were incubated and labelled in the absence of any inhibitor



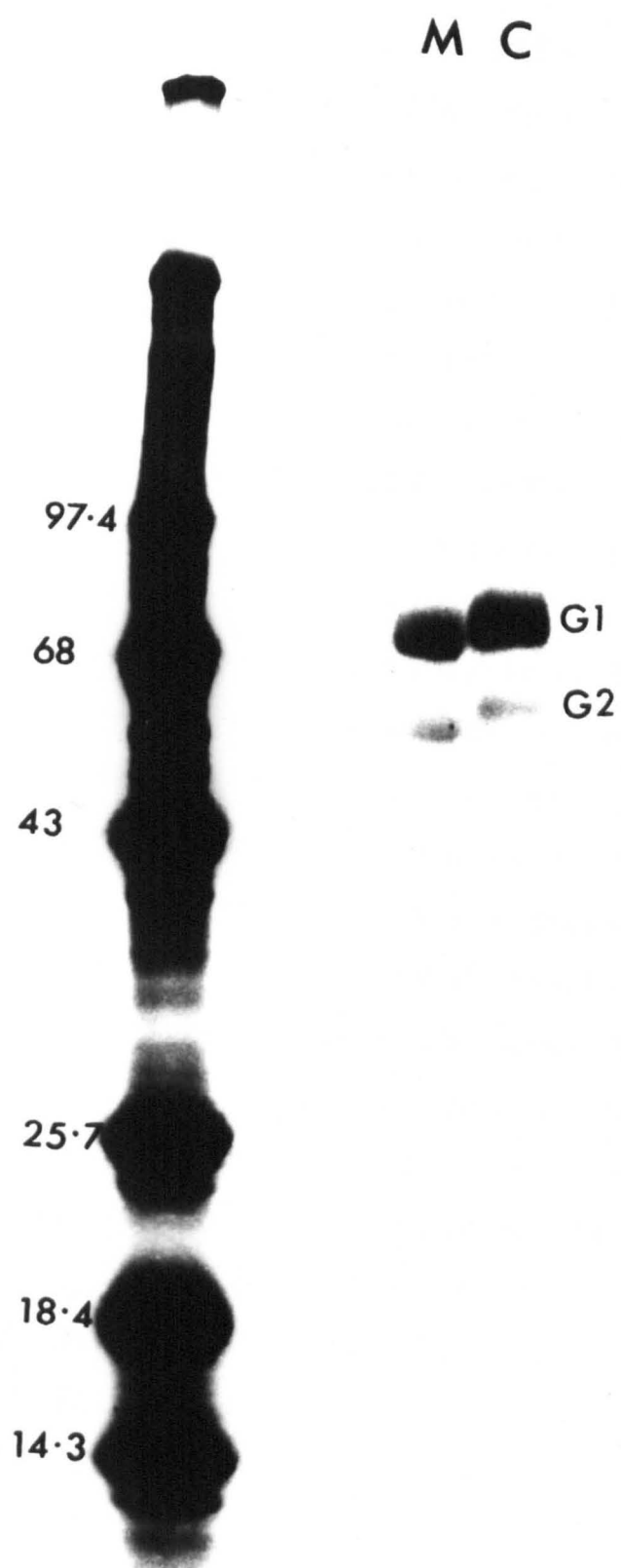
(Figure 55). The insensitivity of glycosylation of the 42K polypeptide to tunicamycin suggests that this polypeptide has non-N-linked oligosaccharides. However it is possible that the band observed in tunicamycin treated cells was G2t which would run in a similar position to the 42K polypeptide. G1t and G2t (the latter polypeptide was only apparent in PEG precipitate samples, Figure 30) were heavily glycosylated indicating that G1 and G2 had a high proportion of oligosaccharides that were not N-linked.

3.3.8. The effect of monensin on [³H]-glucosamine labelled PVM and RS virus polypeptides

Monensin caused a slight increase in mobility of the F related polypeptides of RS virus (Figures 29 and 30), probably due to inhibition of the later stages of the processing of N-linked oligosaccharides. A diffuse region was observed below the G band which may have been due to G partially devoid of O-linked oligosaccharides. The p45 polypeptide band was increased in intensity relative to G as might be expected if this was the precursor to G since this would be the form without O-linked oligosaccharides whose addition was blocked by monensin. The presence of bands in the supernatants is unexpected since monensin would have been expected to block transport of glycoproteins to the cell surface and hence their export to the supernatant. The absence of a clear cut effect of monensin on the glycosylation of this polypeptide suggested that the monensin concentration may not have been sufficiently high

Figure 31 Increase in the mobility of G1 and G2 in monensin treated cells

PVM infected cells were incubated in the presence or absence of monensin ($0.8\mu\text{M}$) for 48 hours after allowing the virus to adsorb. The cells were then labelled for 24 hours with [^3H]-glucosamine and the PVM specific polypeptides immunoprecipitated from the cell lysates with anti PVM serum. The figure shows a fluorograph of the immunoprecipitates analysed by SDS-PAGE on a 6-15 per cent gradient gel.



to block transport and the subsequent O-linked glycosylation.

Polypeptide G2 was slightly reduced in intensity relative to G1 in the samples from monensin treated cells. This could indicate that the effect of monensin was only partial in these experiments and that production of G2 occurs at a location in the cell distal to the site of action of monensin. In some gels even a suboptimal monensin concentration ($0.1\mu\text{M}$) did appear to cause a small increase in mobility consistent with inhibition of the final stages of modification of N-linked oligosaccharides (Figure 31).

3.3.9 Partial proteolysis of G1t and G2t

In order to confirm the suggested relationships between G1t and G2t and G1 and G2 the appropriate bands were excised from gels and subjected to partial proteolysis with chymotrypsin. The results of these digestions are shown in Figure 32. In all the comparisons involving G2t only samples from PEG precipitates were used since this polypeptide was not adequately labelled in immunoprecipitates from cell lysates. Two of five bands observed on digestion of G1t and G2t had similar mobilities, (Figure 32, the bands being indicated by circles). The three differences observed, (indicated by crosses), were due to extra bands obtained on digestion of the larger polypeptide G1t which could have arisen due to sequences or carbohydrate chains present only in this polypeptide or due to digestion of contaminating polypeptides. One of these bands obtained on digestion of

Figure 32 Partial digests of G1t and G2t with chymotrypsin

PVM infected cells were incubated in the presence of tunicamycin (2.5 μ g per ml) and then labelled for 24 hours with [35 S]-methionine (500 μ Ci per ml) in the presence of tunicamycin. Immunoprecipitates using the monoclonal antibody 19/1/C9 of PEG precipitates of the supernatants of these cultures were run on a 6-15 per cent gradient gel. The bands were excised and inserted into the wells of another gel (15 per cent resolving gel with 5cm stacking gel). The proteolysis was carried out in the stacking gel as described in Section 2.5.22. The figure shows a fluorograph of the gel, 0, 100 and 1000 indicating the chymotrypsin concentration used in μ g per ml. Circles indicate peptides common to both G1t and G2t whilst crosses indicate peptides unique to G1t.

G1t
100 1000 1000 100

G2t
0 0

X

O

X

X

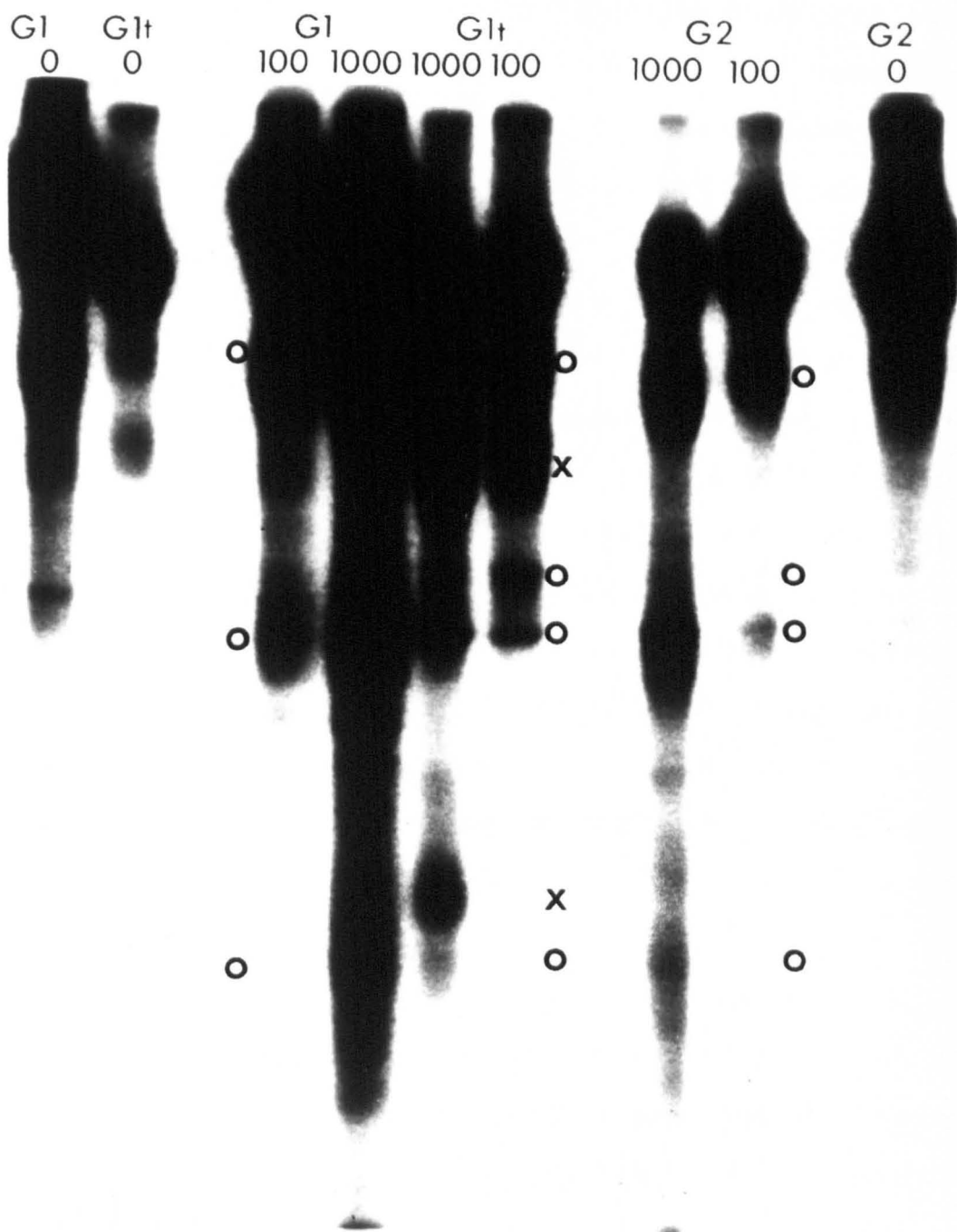
O

O

O

**Figure 33 Partial digestion of G1 and G1t polypeptides with
Chymotrypsin**

G1, G2 and G1t were excised from a gel of PVM polypeptides labelled in the presence or absence of tunicamycin as described in the legend to Figure 32 and immunoprecipitated from cell lysates with anti-PVM serum. The gel slices were inserted into the wells of a second gel and digested during migration through the stacking gel as described in Section 2.5.22. The concentration of chymotrypsin used (0, 100 or 1000 μ g per ml) is indicated above each track. Circles indicate bands common to G1t, G2 and for 3 of the 4 bands G1. Crosses indicate unique bands.



polypeptide G1t had a similar mobility to G2t and so could not be compared with the digestion products of G2t. In Figure 20 it was observed that V8 protease digestion gave a band of greater mobility on digestion of G1t than those obtained on digestion of G1 and G2. This could have been due to the absence of N-linked sugars on the band obtained on digestion of G1t.

A similar comparison of G1t with G1 and G2 was carried out using chymotrypsin to digest the polypeptides (Figure 33). These polypeptides had four bands in common but there were several differences (notably the two polypeptides marked x in the figure). This could have been due to peptides without N-linked sugars being produced from G1t. There was therefore some evidence that G1t and G2t were related to each other and to G1 and G2 but this was not conclusive. The precipitation of these bands by the monoclonal antibody made it appear likely that these bands were related.

3.3.10 Tryptic peptide maps of G1t and G2t

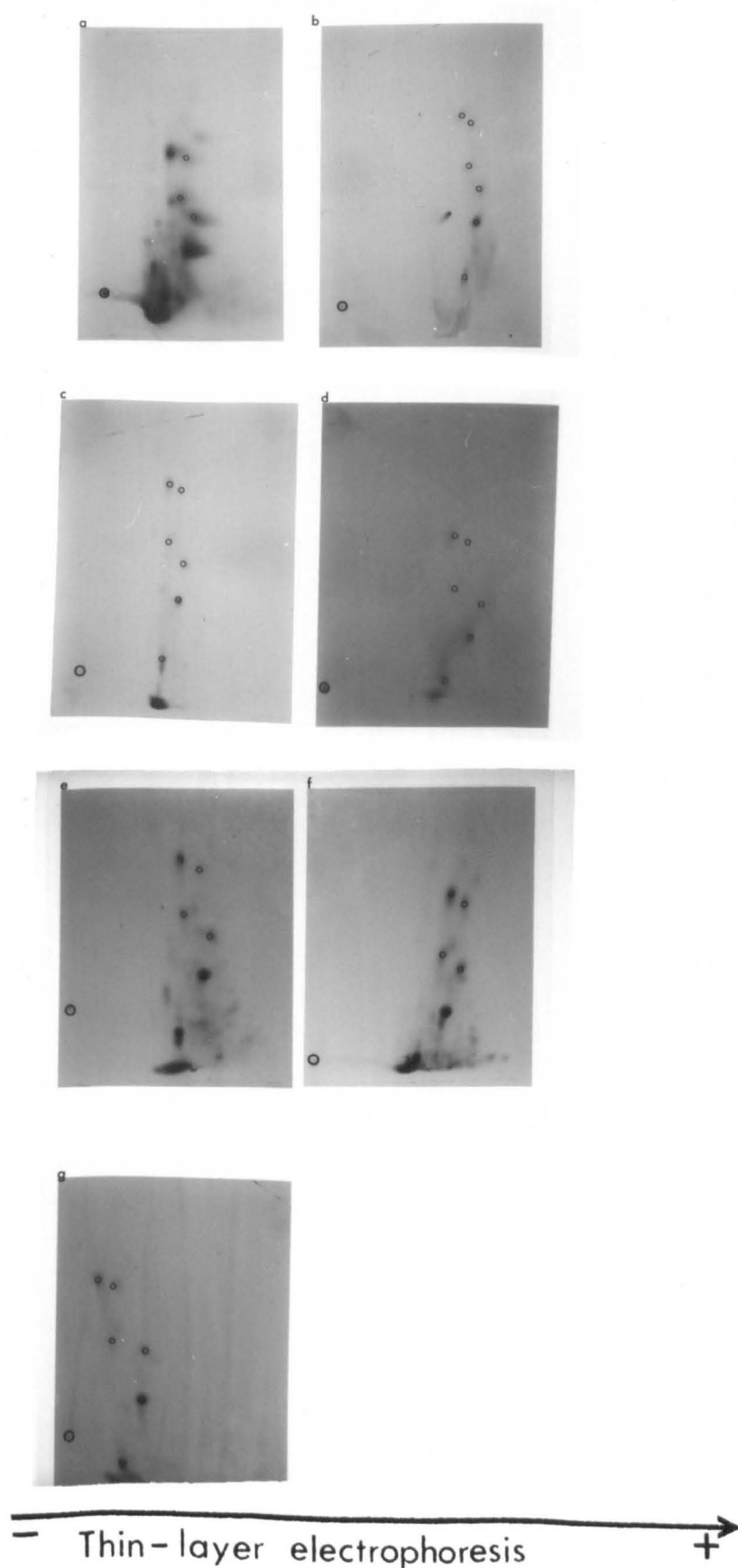
Since the results of partial proteolysis did not give clear cut results with regard to the relationship between G1t, G2t, G1 and G2, tryptic peptide mapping was carried out. The results are shown in Figure 34. Included in this figure are the maps shown previously in Figure 21 of G1 and G2 for comparison with those of G1t, G2t, G1t+G2t, G1t+G1, and G2t+G2. It can be seen that all these maps share spots in common (indicated by small circles). This indicated that

Figure 34 Tryptic peptide maps of G1t and G2t

G1, G2, G1t and G2t were excised from gels of PVM polypeptides labelled with [^{35}S]-methionine (500 μCi per ml) and 4,5- [^3H]-leucine, (750 μCi per ml) and immunoprecipitated with anti-PVM serum. The polypeptides were digested with trypsin as described in Section 2.5.24 and the peptides analysed by two dimensional thin layer electrophoresis/thin layer chromatography. Spots common to all the fluorographs are indicated by small circles. Large circles indicate the points of application of the samples.

- (a) Fluorograph of a tryptic peptide map of G1.
- (b) Fluorograph of a tryptic peptide map of G2.
- (c) Fluorograph of a tryptic peptide map of G1t.
- (d) Fluorograph of a tryptic peptide map of G2t.
- (e) Fluorograph of a tryptic peptide map of mixed G1t and G2t peptides.
- (f) Fluorograph of a tryptic peptide map of mixed G1 and G1t peptides.
- (g) Fluorograph of a tryptic peptide map of mixed G2 and G2t peptides.

TLC



these polypeptides were in fact related. Other spots not indicated by circles could not be identified on some fluorographs due to insufficient label incorporation into the peptides or insufficient resolution of the spots. The large circles at the bottom left hand corner indicate the point of application of the sample. Material at the bottom of the fluorographs probably represents glycopeptides.

3.3.11 Effect of protease inhibitors on glycoprotein processing

The results of the pulse chase experiment described in Section 3.3.4 suggested that G2 was derived from G1 by a post-translational event. Proteolytic cleavage would provide a mechanism by which G1 could be modified to give G2. Phenylmethanesulphonyl fluoride was routinely used in the buffers used during the preparation of samples but it could be that the change had occurred during the labelling period when no inhibitor was present or that the protease involved was not inhibited by this inhibitor. If the proteolytic cleavage occurred intracellularly the protease inhibitors would probably not be effective so this possibility could not be excluded. Since G2 became relatively abundant in the supernatant it was possible that at least part of the processing continued after release of virus or protein from the cells and that the G2 polypeptide occurring in the cell lysates could have been due to proteolytic cleavage during the immunoprecipitation procedure.

To study these possibilities samples were prepared

Figure 35 Effect of α_2 -macroglobulin on PVM polypeptides identified by immunoprecipitation

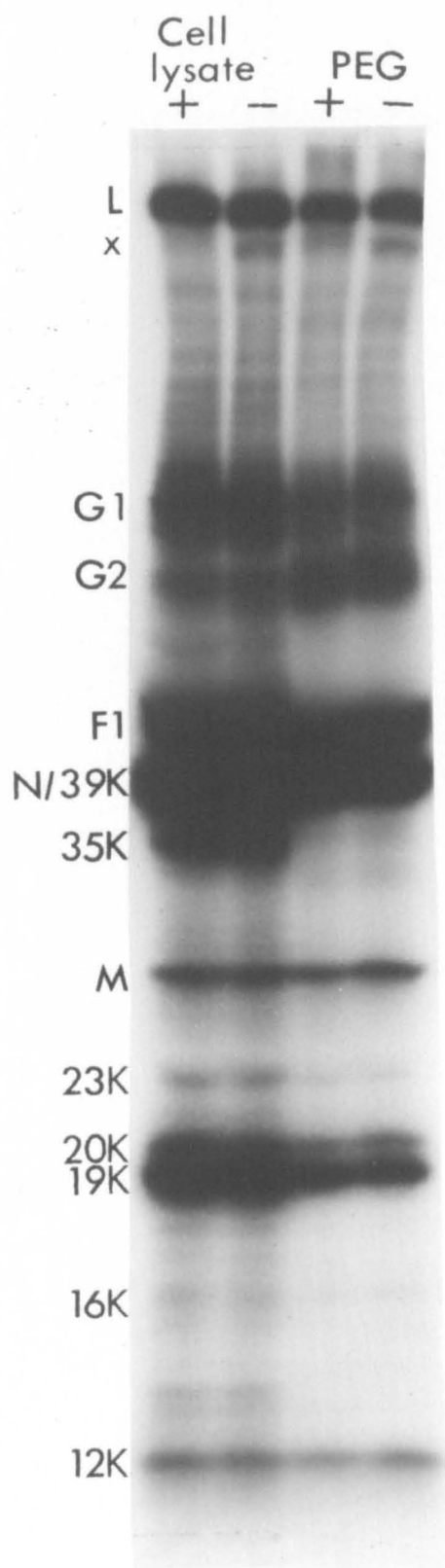
PVM infected BS-C-1 cells were labelled with [35 S]-methionine (50 μ Ci per ml) for 24 hours in the presence or absence of α_2 -macroglobulin. Cell lysates and PEG precipitates of the supernatants were then immunoprecipitated with anti-PVM serum in the presence or absence of α_2 -macroglobulin. The figure shows a fluorograph of the immunoprecipitates analysed by SDS-PAGE using a 6-15 per cent gradient gel. The PVM specific polypeptides are indicated on the left, x indicating a polypeptide only observed when the protease inhibitor was not used.

Cell lysate Samples immunoprecipitated from cell lysates.

PEG Samples immunoprecipitated from PEG precipitates of cell supernatants.

+ Samples prepared with α_2 -macroglobulin present throughout labelling and immunoprecipitation

- Samples prepared without any protease inhibitors being present.



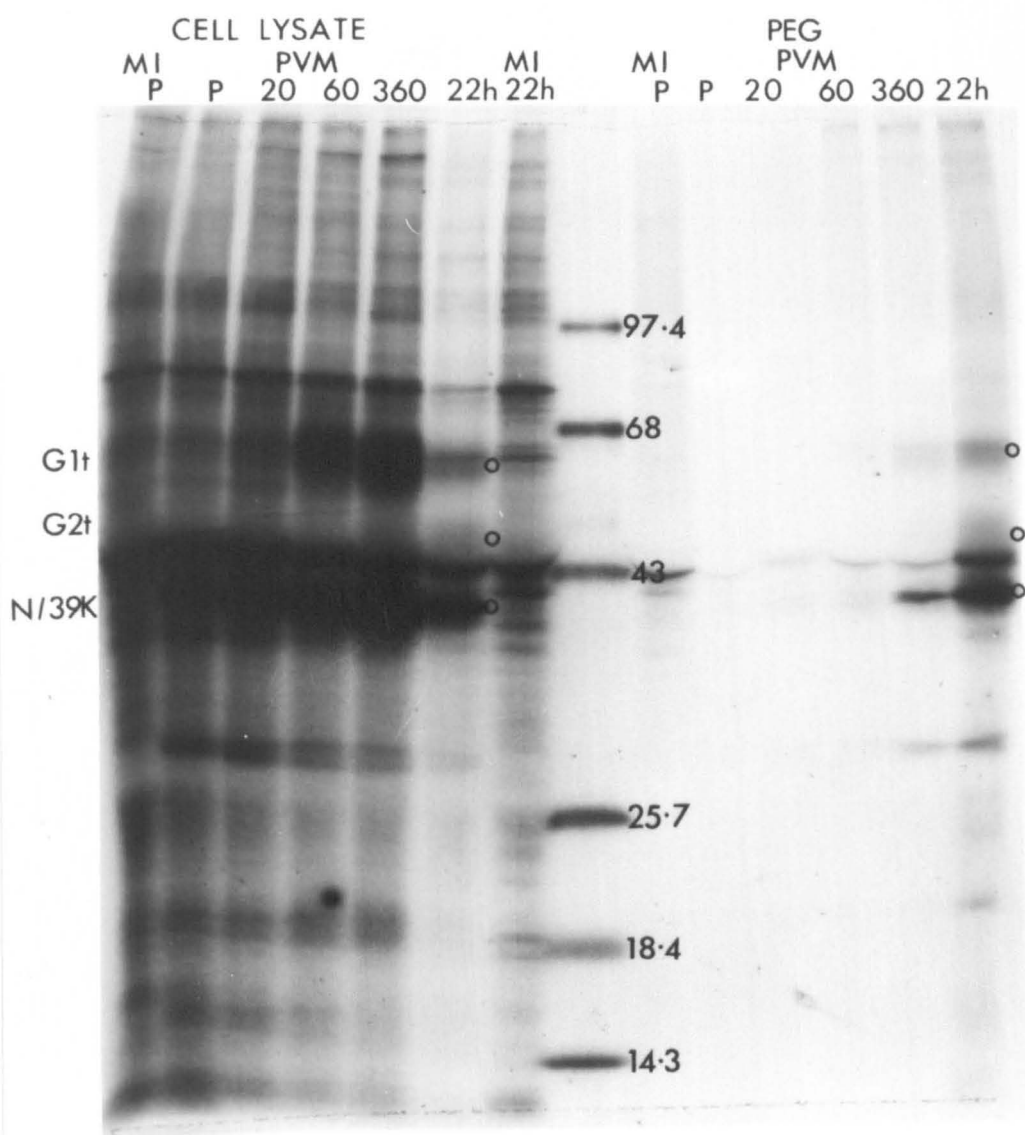
without any protease inhibitors and with α_2 -macroglobulin present throughout the labelling and immunoprecipitation steps. This protease inhibitor has been shown to inhibit all endoproteases tested (Barrett & Starkey, 1973). The result is shown in Figure 35. It was observed that the only apparent difference between the two sets of samples was the presence of a band just below L when no protease inhibitor was used which was absent when the protease inhibitor was used. This suggested that only the L polypeptide was affected by proteases during these experiments. It would appear therefore that the conversion of G1 to G2 was unlikely to have been due to extracellular proteolytic activity or proteolytic activity during sample preparation. The possibility that an α_2 -macroglobulin insensitive protease existed or that the concentration used ($10\mu\text{gml}^{-1}$) was not sufficient to inhibit the proteases present cannot be excluded.

3.3.12 Relationship of G1t and G2t in pulse chase experiments.

The most likely relationship of G1t and G2t to G1 and G2 would be that G1t represented G1 without its N-linked polypeptides and G2t represented G2 without its N-linked polypeptides as suggested earlier (Section 3.3.5). If this was the case it could be predicted by comparison with the relationship between G1 and G2 that G2t would be derived from G1t in pulse chase experiments. The result of such an experiment is shown in Figure 36. As predicted G1t appeared

Figure 36 G related polypeptides observed during a pulse chase experiment performed in the presence of tunicamycin.

PVM or mock infected cells were pulse labelled for 10 minutes with [^{35}S]-methionine (400 μCi per ml) 48 hours after infection and were then incubated in the absence of labelled methionine for various times as described in Section 2.5.13. Cell lysates and PEG precipitates of cell supernatants were immunoprecipitated with the monoclonal antibody 19/1/C9. The figure shows a fluorograph of these samples analysed by SDS-PAGE on a 6-15 per cent gradient gel. Samples immunoprecipitated from cell lysates are labelled cell lysate and those immunoprecipitated from PEG precipitates of cell supernatants are labelled PEG. Samples immunoprecipitated from mock infected cell samples are indicated by MI above the track, all the other tracks show immunoprecipitates of PVM infected cells. The length of the chase periods is indicated in minutes except for the 22 hour (22h) chase period. P indicates that the samples were harvested immediately following the pulse period. M_r s ($\times 10^{-3}$) are indicated adjacent to the molecular weight standards. PVM specific polypeptides are indicated by circles to the right of the tracks and labelled on the left.



first, (as in the pulse chase experiment with untreated PVM infected cells no precursor was observed), after a chase period of about 60 minutes, followed by G2t which was not clearly visible until after a 22 hour chase period. Increasing amounts of the N/39K polypeptide with increasing chase periods was probably due to an association of the N polypeptide with other virus polypeptides that occurred under the conditions used for the immunoprecipitation as is discussed later (Section 3.4.4).

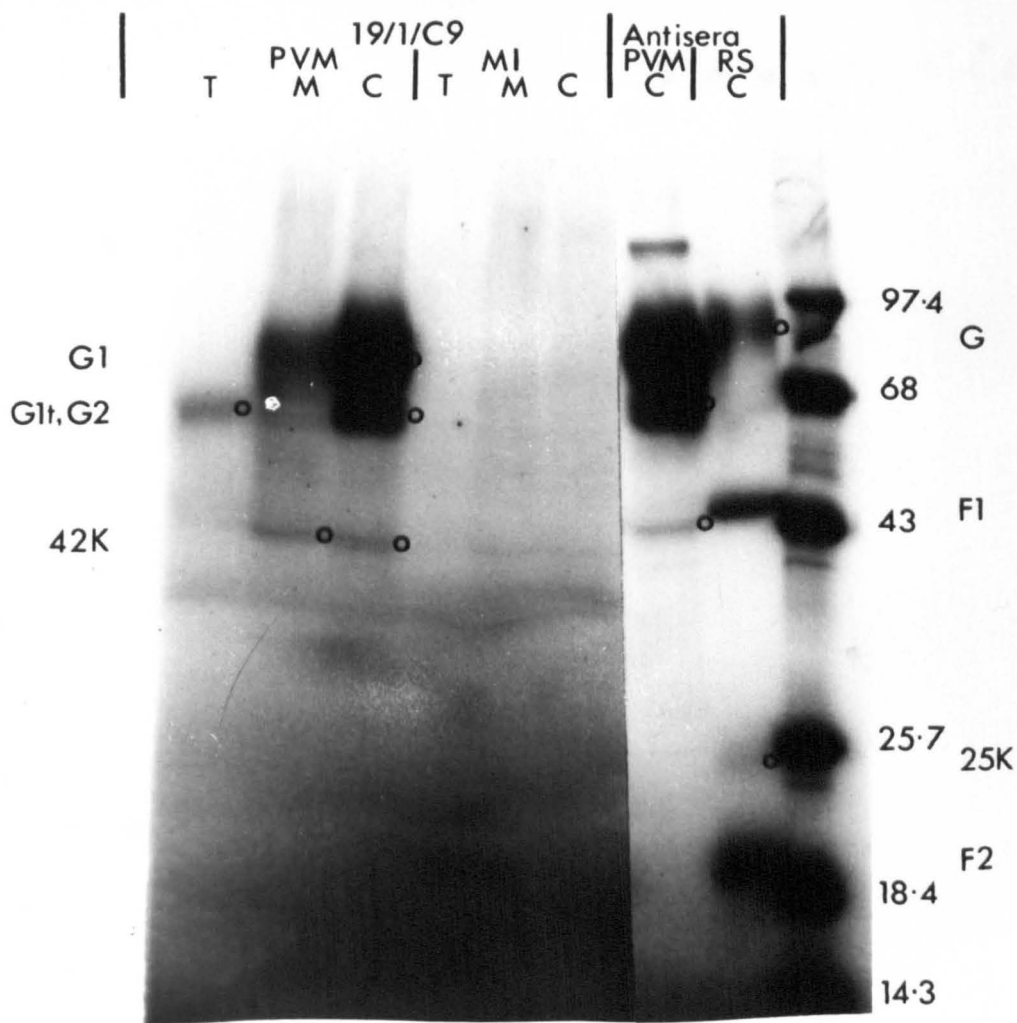
3.3.13 Demonstration of a 42K glycoprotein related to G

Two glycosylated polypeptides with M_r 's of 44K and 42K were described in Section 3.1.5. To determine if either of these polypeptides was related to the G proteins [^3H]-glucosamine labelled lysates of PVM infected cells were immunoprecipitated with the monoclonal antibody 19/1/C9. The samples were run on the same gel as samples immunoprecipitated with the anti-PVM serum in order to distinguish between the 42K and 44K polypeptides. The results are shown in Figure 37, the exposure of the polypeptides immunoprecipitated by the antiserum being too short to reveal the 44K polypeptide. A polypeptide was immunoprecipitated by the monoclonal antibody that co-migrated with the 42K polypeptide. The low intensity of this band meant that the possibility that the 44K polypeptide was also related to the G protein could not be excluded.

**Figure 37 [³H]-glucosamine labelled polypeptide
immunoprecipitated by the anti G monoclonal antibody**

The monoclonal antibody 19/1/C9 was used to immunoprecipitate [³H]-glucosamine labelled polypeptides from PVM and mock infected cell lysates. Labelling was carried out in the presence or absence of tunicamycin (2.5 μgml^{-1}) or monensin (0.8 μM). [³H]-glucosamine labelled PVM and RS virus polypeptides immunoprecipitated with the homologous antisera were run on the same gel as controls.

19/1/C9-	Samples immunoprecipitated with monoclonal antibody 19/1/C9
Antisera-	Samples immunoprecipitated with the homologous antisera
PVM-	Samples immunoprecipitated from PVM infected cell lysates
RS-	Samples immunoprecipitated from RS virus infected cell lysates
MI-	Samples immunoprecipitated from mock infected cell lysates
T-	Samples immunoprecipitated from lysates of tunicamycin treated cells
M-	Samples immunoprecipitated from lysates of monensin treated cells
C-	Samples Immunoprecipitated from lysates of untreated cells



3.3.14 Demonstration of a possible F like glycoprotein by labelling with [³H]-glucosamine.

The results described in Sections 3.3.2 and 3.3.3 showed that the two glycoproteins of PVM described by others (Cash et al., 1979a) represented two forms of a single protein. This protein probably represented the haemagglutinin since haemagglutination was inhibited by the monoclonal antibody directed against the G1/G2 protein. Other members of the family *Paramyxoviridae* possess two major glycoproteins the haemagglutinin or haemagglutinin-neuraminidase in paramyxoviruses or the G glycoprotein in RS virus and a fusion protein. The latter protein is typically cleaved to give a large and a small fragment which are linked by disulphide bonds. PVM showed two glycosylated bands in the 45K region of the gel (the 44K and 42K polypeptides which were described in Section 3.1.5). In order to establish if there was an F like protein in this region of the gel samples labelled with [³H]-glucosamine and immunoprecipitated with anti-PVM serum were run under reducing and non-reducing conditions along with the RS F protein as a control. The result is shown in Figure 38. It was found that the bands in the 45K region were reduced in intensity under non-reducing conditions and a band of lower mobility was observed (F1,2). The band labelled F1 may represent some reduced F1 or a glucosamine labelled band in this region of the gel that is not related to the F protein. No evidence of a glycosylated F2 like polypeptide was

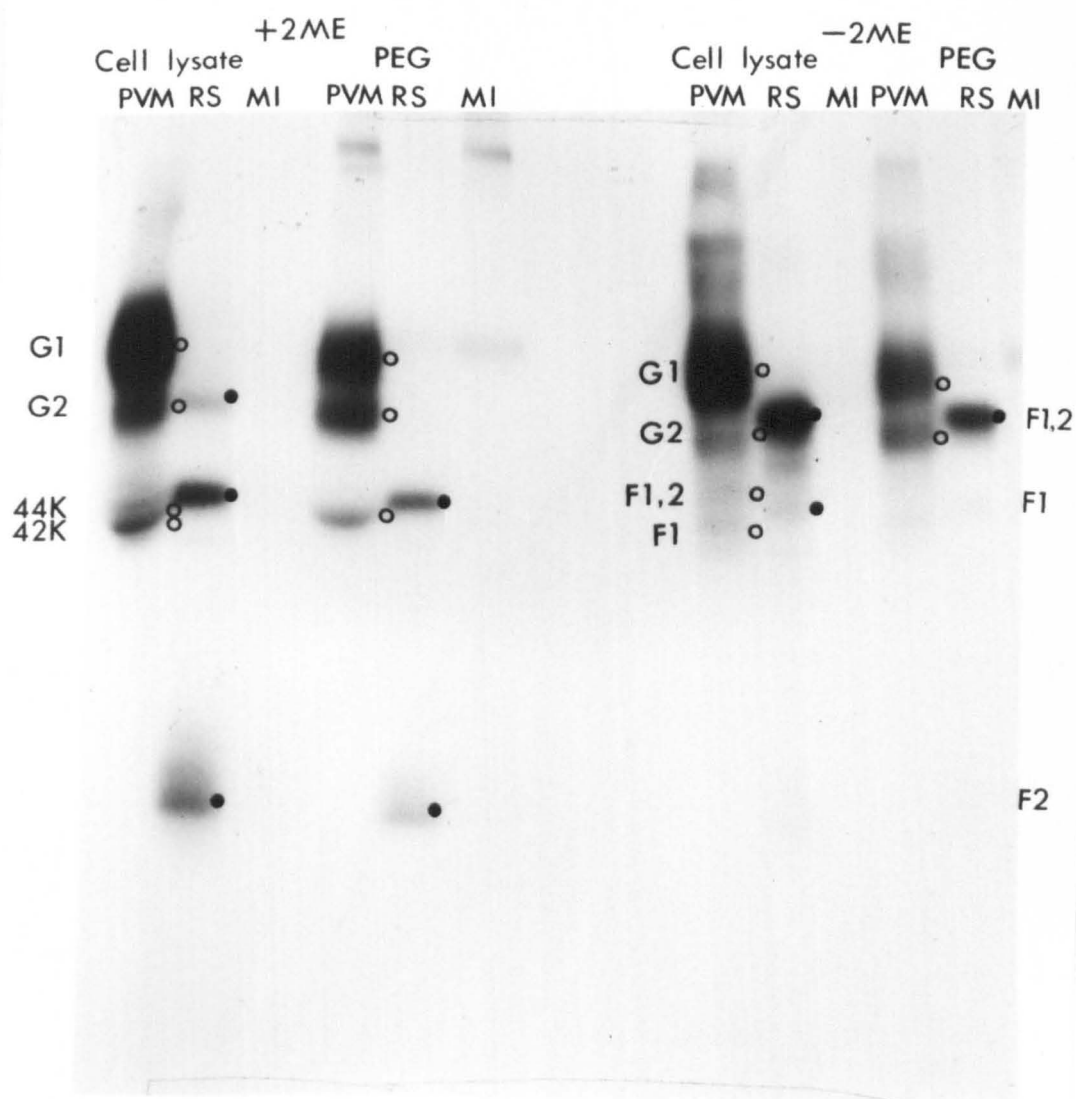
**Figure 38 [³H]-glucosamine labelled PVM polypeptides
observed under reducing and non-reducing conditions**

PVM infected RS virus infected and mock infected cells were incubated for 48 hours at 33°C and then labelled for 24 hours with [³H]-glucosamine (100μCi per ml). Cell lysates and PEG precipitates of the supernatants were immunoprecipitated using anti-PVM serum for the samples from PVM and mock infected cells and the anti-F monoclonal antibody 43.1 for the samples from RS virus infected cells. The immunoprecipitates were divided into two parts and one part boiled in sample buffer containing 2-mercaptoethanol (+2ME) and the other in sample buffer lacking 2-mercaptoethanol (-2ME). A fluorograph of the samples analysed by SDS-PAGE using a 6-15 per cent gradient gel is shown in the figure. Samples immunoprecipitated from cell lysates are labelled cell lysate whilst those immunoprecipitated from PEG precipitates of cell supernatants are labelled PEG. The PVM specific polypeptides are labelled on the left and the RS virus specific polypeptides are labelled on the right.

PVM Samples derived from PVM infected cells

RS Samples derived from RS virus infected cells

MI Samples derived from mock infected cells



obtained.

3.3.15 Identification of an F like polypeptide by immunoprecipitation of polypeptides expressed on the cell surface

It was possible that the F like polypeptide produced in PVM infected cells was poorly glycosylated and could be more readily labelled with [^{35}S]-methionine. A candidate for such a protein existed in the form of the F1 polypeptide described in Section 3.1.4 which migrated in a similar position to the F1 polypeptide of RS virus. Virus envelope glycoproteins are expressed at the cell surface so it was of interest to find out which PVM polypeptides were expressed on the surface of infected cells. To do this the approach of Morrison & Ward (1984) was used. PVM infected cells labelled with [^{35}S]-methionine were incubated with antibody, the unreacted antibody washed off, the cells lysed and the antibody adsorbed with Immuno-Precipitin which could then be washed and the proteins eluted in the usual way. Only proteins present on the cell surface would be expected to be immunoprecipitated. The results of such an experiment are shown in Figure 39. Surprisingly relatively little of the G1 or G2 polypeptides were ever detected on the cell surface in these experiments despite the strong cell surface immunofluorescence that was observed using the monoclonal antibody recognizing this polypeptide. This was probably due to the poor labelling that was obtained with this

Figure 39 Immunoprecipitation of PVM polypeptides expressed on the cell surface

PVM infected RS virus infected and mock infected cells were labelled with [³⁵S]-methionine as described in Section 3.5.12. Polypeptides were immunoprecipitated from the cell surface by incubation of the cells with a 100 fold dilution of ascitic fluid in PBS for 30 minutes on ice and washing five times with PBS prior to lysis of the cells as described in Section 2.5.12. The antibody-antigen complexes were precipitated using Immuno-Precipitin as described in Section 2.5.15. PVM polypeptides were then immunoprecipitated from the remaining lysate. One half of each immunoprecipitate was boiled in sample buffer with 2-mercaptoethanol (+2ME) and the other half boiled in sample buffer without 2-mercaptoethanol (-2ME). The figure shows a fluorograph of the immunoprecipitates analysed by SDS-PAGE using a 6-15 per cent gradient gel. The PVM specific polypeptides are labelled on the left and the RS virus specific polypeptides are labelled on the right.

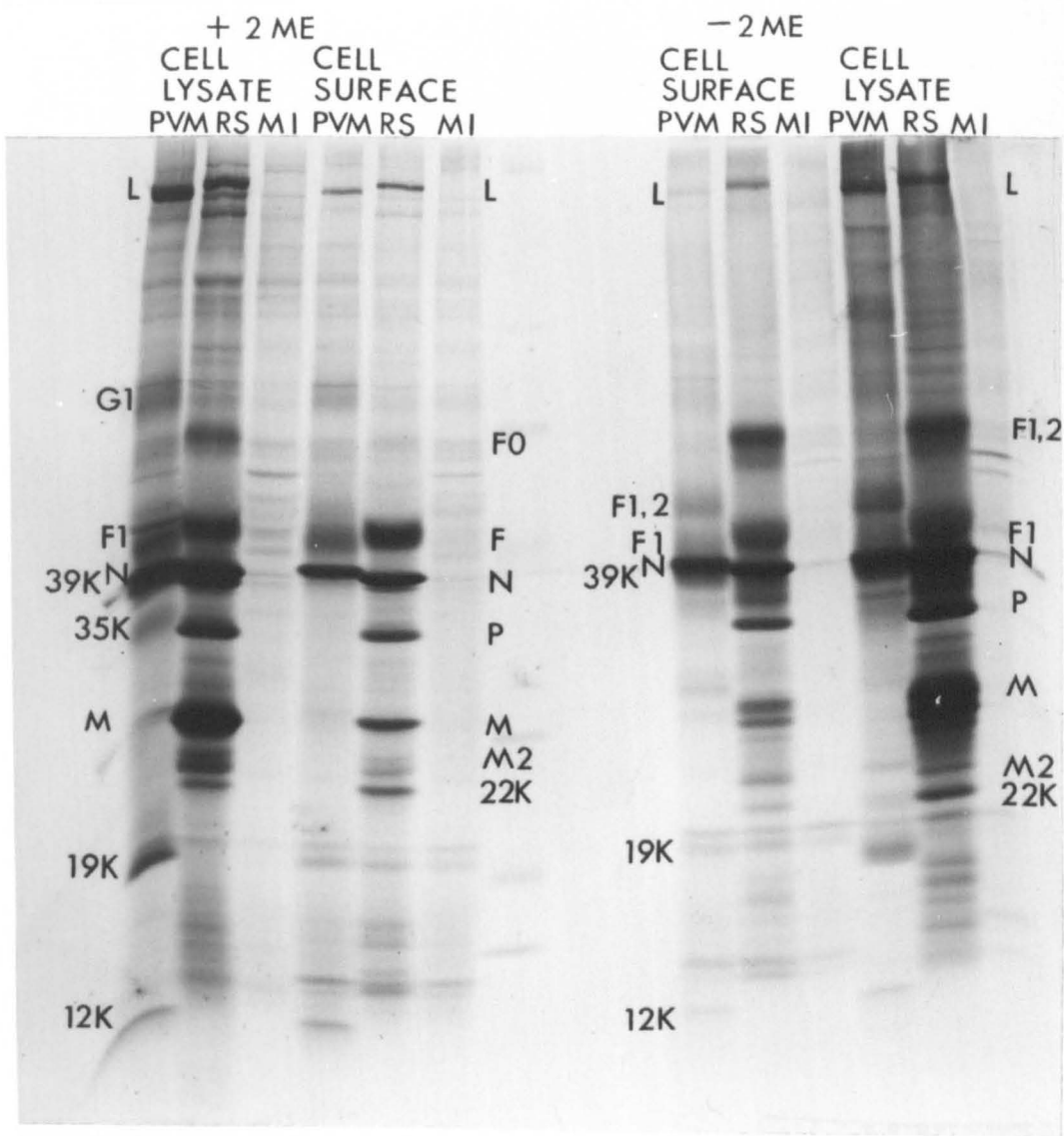
Cell lysate Polypeptides immunoprecipitated from cell lysates

Cell surface Polypeptides immunoprecipitated from the surface of infected cells.

PVM Polypeptides immunoprecipitated from PVM infected cells using anti-PVM serum

RS Polypeptides immunoprecipitated from RS virus infected cells using anti-RS virus serum

MI Polypeptides immunoprecipitated from mock infected cells using anti-PVM serum.



polypeptide. Two major bands were obtained in cell surface immunoprecipitates, N/39K and F1. The polypeptide designated F1 showed some similarity to the F1 polypeptide of RS virus, becoming reduced in intensity if non-reducing conditions were used with a concomitant increase in the intensity of a 50K polypeptide designated F1,2 by analogy with the RS F1,2 polypeptide observed under the same conditions.

Precipitation of the N/39K polypeptide was later shown to be an artefact (Section 3.4.4), not being immunoprecipitated if a lower salt concentration was used in the immunoprecipitation buffer. The 39K polypeptide was also not detected by FACS analysis using the anti-39K protein monoclonal antibody 26/404 (Section 3.4.3). An F2 like polypeptide could not be clearly identified, possible candidates would be the 9K polypeptide observed occasionally (Section 3.1.2) and the 12K polypeptide. The latter polypeptide was observed to be expressed on the cell surface but was not clearly reduced in intensity under non-reducing conditions. Both of these polypeptides would appear to be too large to account for the mobility difference between F1,2 and F1 although this could have been due to aberrant migration. Attempts to demonstrate a polypeptide of the predicted size for F2, (M_r of 5,000) using ^{14}C labelled protein hydrolysate and the gel system of Swank & Munkres (1971) failed although this could have been due to insufficient label incorporation. On the gel shown in Figure 39 the RS virus infected cells were probably disrupted during the cell surface immunoprecipitation since all of the

**Figure 40 The effect of tunicamycin on PVM and RS virus
polypeptides expressed on the cell surface**

PVM and RS virus infected cells were incubated for 48 hours at 33°C in the presence or absence of tunicamycin (2.5µg per ml) or monensin (0.8µM) and then labelled for 24 hours with [³⁵S]-methionine (50µCi per ml). The virus specific polypeptides present on the cell surface and in total cell lysates were immunoprecipitated by homologous antisera as described in the legend to Figure 39. An immunoprecipitation of the cell surface polypeptides was also carried out in the absence of antibody. A fluorograph of the gel of the immunoprecipitates of cell surface and cell lysate polypeptides is shown in the figure.

PVM- Polypeptides immunoprecipitated from samples of PVM infected cells.

RS- Polypeptides immunoprecipitated from samples of RS virus infected cells.

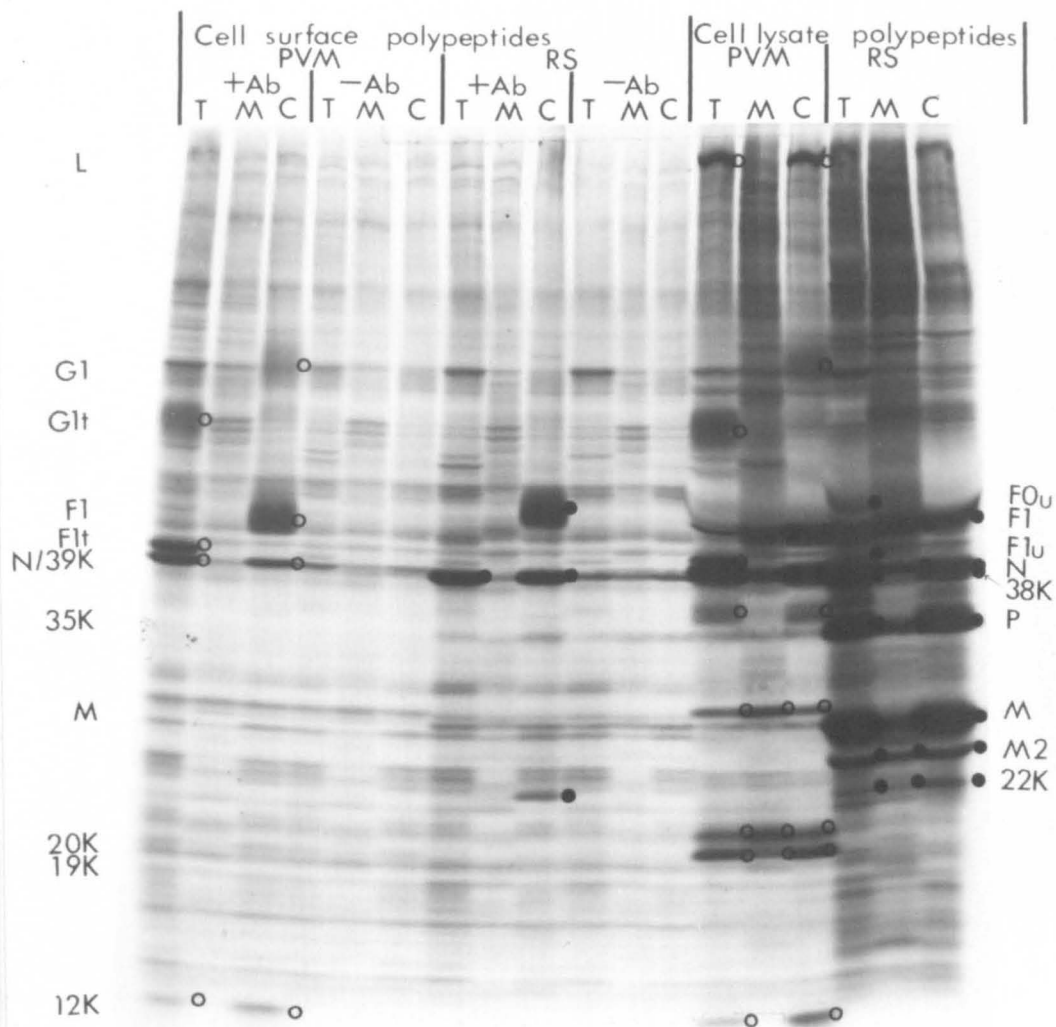
+Ab- Immunoprecipitation carried out with homologous antiserum.

-Ab- Control immunoprecipitation carried out without antibody.

T- Cells were incubated in the presence of tunicamycin.

M- Cells were incubated in the presence of monensin.

C- Cells were incubated in the absence of glycosylation inhibitors.



RS polypeptides could be observed in the cell surface immunoprecipitation sample. These results suggested that there was an F like polypeptide in PVM that was poorly glycosylated.

3.3.16 Effect of tunicamycin on PVM and RS polypeptides expressed on the cell surface.

Figure 40 shows the effect of tunicamycin and monensin on polypeptides of PVM and RS virus that were expressed on the surface of infected cells. The effect of monensin is described in the following section. The RS G polypeptide was not identified since the samples were labelled with [^{35}S]-methionine and this polypeptide has a low methionine content. The RS virus F related proteins observed in the presence of inhibitors were not expressed at very high levels on the surface of infected cells. The unglycosylated forms of F0 and F1, (F0_u and F1_u) were observed in immunoprecipitates of total cell lysates but not to a very great extent in the cell surface samples. The reason for this was not clear and the result differs from that observed with the forms of the PVM glycoproteins synthesized in the presence of tunicamycin described below.

Cells infected with PVM and labelled in the presence of tunicamycin showed two noticeable differences from control PVM infected cells. G1 and F1 were not apparent whilst two new bands, G1t and F1t were observed. Both of the polypeptides were expressed on the surface of infected cells. Since G1t was known to be related to G1 (Sections

3.3.9 and 3.3.10), the presence of F1t and absence of F1 in tunicamycin treated cells suggested that F1t was an unglycosylated form of F1 and that despite the apparent absence of an F like polypeptide in [³H]-glucosamine labelled gels there was an F1 polypeptide containing N-linked sugars. The difference in mobility between the F1 and F1t polypeptides of PVM was less than that between the F1 and F1u polypeptides of RS virus suggesting that F1 in PVM was less highly glycosylated than F1 in RS virus. Whatever the nature of the 12K polypeptide expressed on the cell surface it did not appear to have N-linked sugars.

3.3.17 The effect of monensin on PVM and RS virus polypeptides expressed on the cell surface

PVM and RS polypeptides expressed on the surface of cells treated with monensin are shown in Figure 40. There was a slight increase in the mobility of the F1 protein of RS virus observed in cell lysates and this polypeptide was not observed on the cell surface. No PVM specific polypeptides could be detected on the surface of monensin treated PVM infected cells and only a few faint bands in the F1 region were observed in the immunoprecipitated cell lysate these possibly being due to forms of F1 showing heterogeneous glycosylation. The L, G1, N, 35K and 12K polypeptide bands were also reduced in intensity in monensin treated cells. The expression of the M, 20K and 19K polypeptides was relatively unaffected by monensin treatment.

**Figure 41 Partial proteolytic digestion of F1 and F1t with
chymotrypsin**

PVM infected cells were incubated for 48 hours at 33°C in the presence or absence of tunicamycin (2.5µg per ml) and then labelled for 24 hours with [³⁵S]-methionine (500µCi per ml). The polypeptides were immunoprecipitated from the cell lysates with anti-PVM serum and run on a 6-15 per cent gradient gel. The F1 and F1t polypeptide bands were excised from the gel and digested during stacking in a second gel as described in Section 2.5.22. The resolving gel of the second gel was a 15 per cent acrylamide single concentration gel. The figure shows a fluorograph of this gel, the concentration of chymotrypsin used being indicated above each track in µg per ml. The circles indicate bands common to both digests whilst the crosses indicate bands unique to the digest of one of the polypeptides.

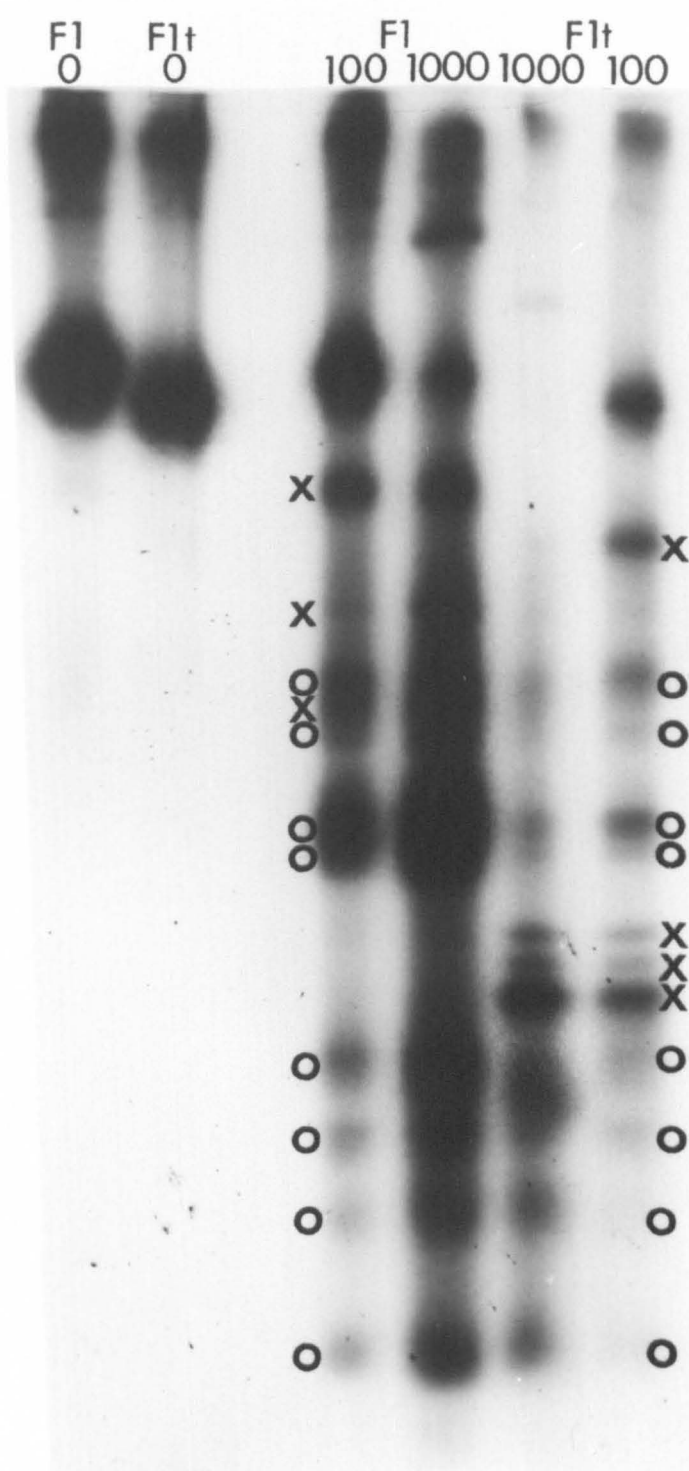
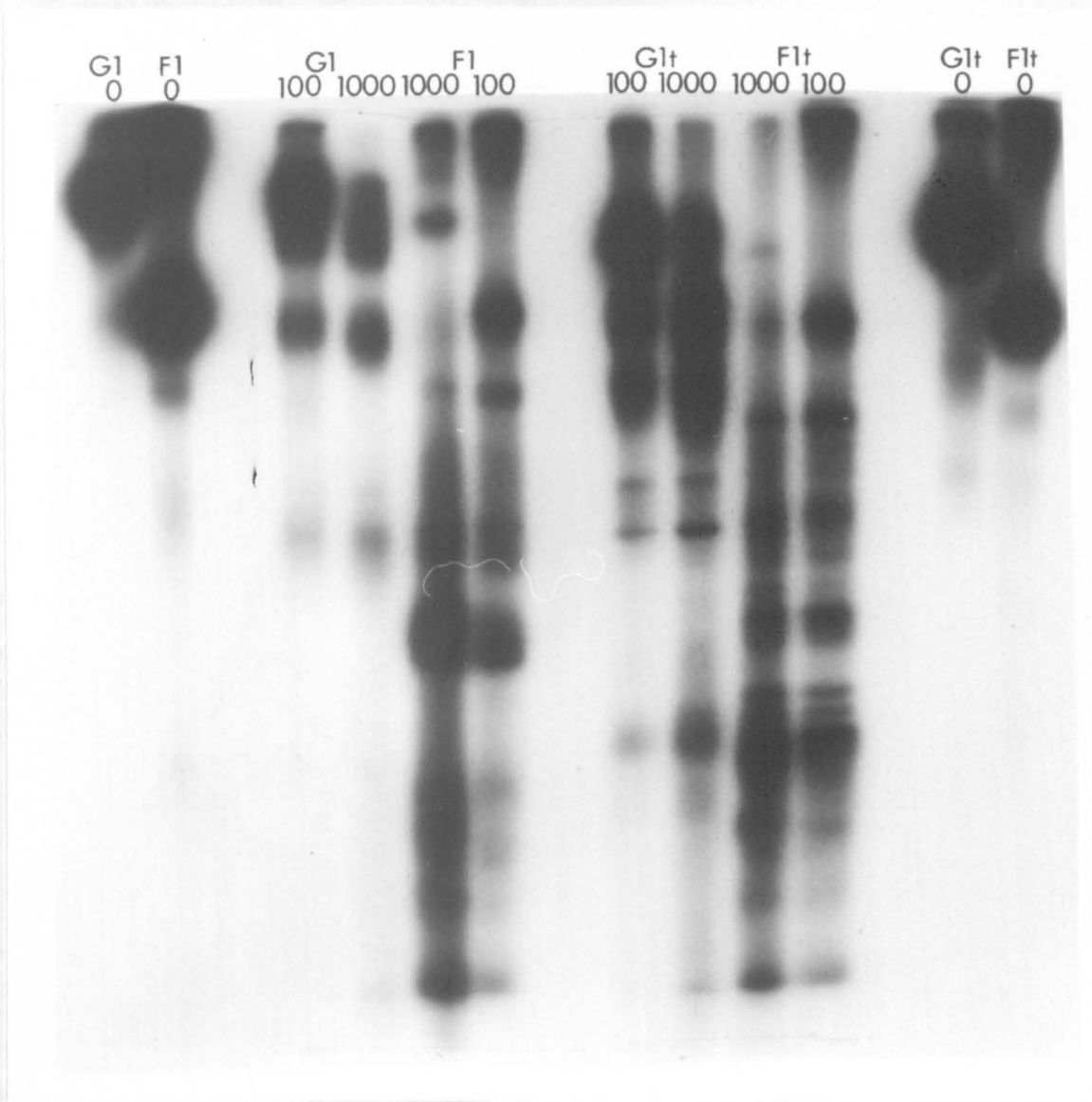


Figure 42 Comparison of partial proteolytic digests of G1,
F1, G1t and F1t

PVM infected cells were incubated for 48 hours at 33°C in the presence or absence of tunicamycin (2.5 µg per ml) and then labelled for 24 hours with [³⁵S]-methionine (500 µCi per ml). The polypeptides were immunoprecipitated from the cell lysates with anti-PVM serum and run on a 6-15 per cent gradient gel. The G1, F1, G1t and F1t polypeptide bands were excised from the gel and digested during stacking in a second gel as described in Section 2.5.22. The resolving gel of the second gel was a 15 per cent acrylamide single concentration gel. The figure shows a fluorograph of this gel, the concentration of chymotrypsin used being indicated above each track in µg per ml.



3.3.18 Partial proteolysis of the F related polypeptides

Comparison of digests of F1 and F1t with chymotrypsin (Figure 41), revealed several differences between the polypeptides (indicated by crosses), perhaps due to glycosylated peptides in the digest of F1 or an increase in the sensitivity of the non-glycosylated polypeptide to digestion. These differences do not rule out a relationship between these polypeptides since there were eight bands common to digests of both polypeptides but they did not provide clear evidence for such a relationship. Figure 42 shows that there was no similarity between the F and G related polypeptide digests suggesting that these polypeptides were distinct.

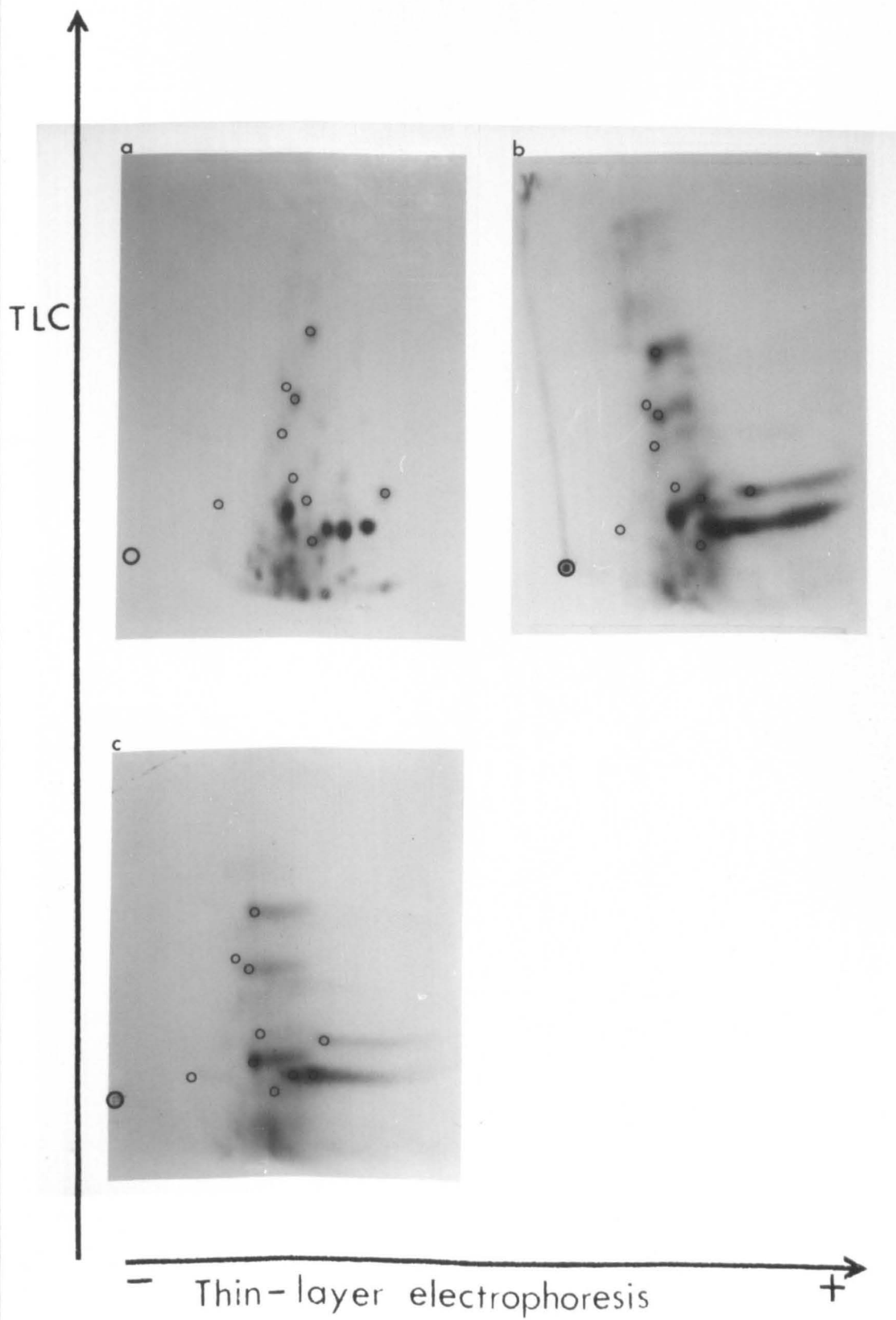
3.3.19 Tryptic peptide maps of F related polypeptides

F1 and F1t were not conclusively shown to be related by the partial proteolysis experiments described above so they were subjected to analysis by two dimensional tryptic peptide mapping. The results obtained with digests of F1, F1t and a mixture of the two digests are shown in Figure 43. In the case of the mixture poor resolution was obtained in the first dimension so not all the spots could be resolved. It can be seen that the three patterns show considerable similarity and the two polypeptides were therefore related. The unlabelled spots were not adequately labelled or resolved on some of the fluorographs to determine whether or not they were common to all the peptide maps. The point of

Figure 43 Tryptic peptide maps of PVM F related polypeptides

PVM infected cells were labelled for 48 hours in the presence or absence of tunicamycin (2.5 μ g per ml) and then labelled for 24 hours with [35 S]-methionine (500 μ Ci per ml) and 4,5- [3 H]-leucine (750 μ Ci per ml). The PVM specific polypeptides were immunoprecipitated with anti-PVM serum and separated by SDS-PAGE using a 6-15 per cent gradient gel. The F1 and F1t bands were excised and digested with trypsin as described in Section 2.5.24. The peptides were analysed by two dimensional thin layer electrophoresis/thin layer chromatography. Fluorographs of the tlc plates are shown in the figure. Spots common to all the fluorographs are indicated by small circles. A large circle on each fluorograph indicates the point at which the samples were applied.

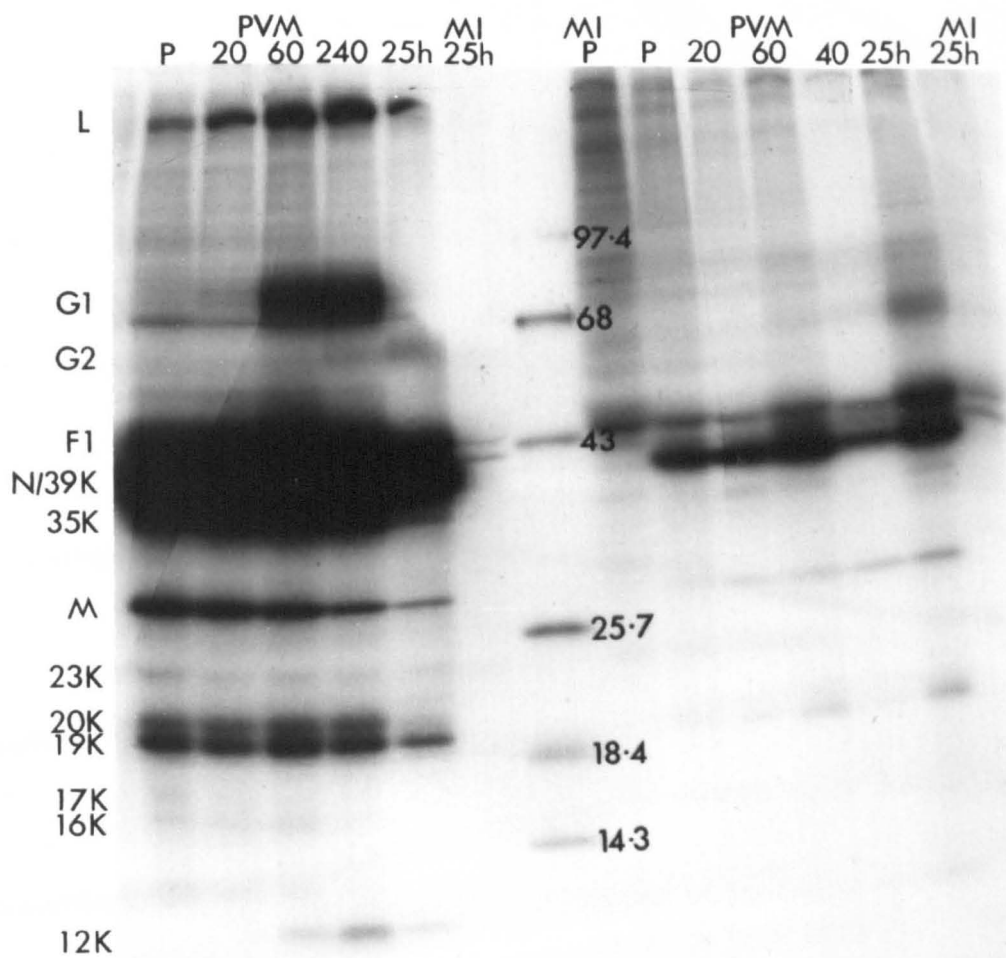
- (a) Fluorograph of chromatograph of tryptic peptide digest of F1.
- (b) Fluorograph of chromatograph of tryptic peptide digest of F1t.
- (c) Fluorograph of tryptic peptide digests of F1 and F1t analysed on the same chromatograph.



application of the sample is indicated by a large circle.

3.3.20 Pulse chase experiment using anti-PVM serum

No antibody specific for the the PVM F protein was available so studies on the F protein had to be carried out using the murine anti-PVM serum. The results of a pulse chase experiment carried out using this serum are shown in Figure 44. Most of the polypeptides showed a decreasing intensity in the cell lysate as the chase periods became longer with a corresponding increase in intensity of the polypeptide band in the PEG precipitate samples. This would be expected to occur as the polypeptides were incorporated into the virus. There did appear to be some label incorporation during the early chase periods in this gel, the L polypeptide becoming more heavily labelled up to 60 minutes of chase but this could not account for the more abrupt appearance of G1 and the 12K polypeptide. The 35K polypeptide did not alter much in intensity during the chase periods until after a 25 hour chase period when this band was fainter. The fate of this polypeptide was not clear since it has not been observed in polyethylene glycol precipitates of culture supernatants and there was no obvious increase in intensity of any other band at this time. It may have simply been degraded or changed to a form that was obscured by other bands. The significance of a 17K polypeptide observed only after the pulse period was not clear. A 17K polypeptide was observed *in vitro* so this may represent a primary gene product although it is not possible



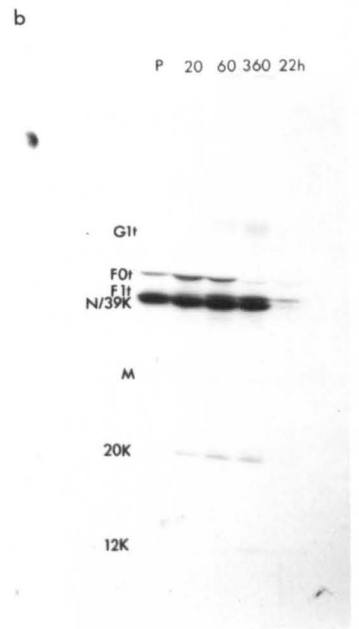
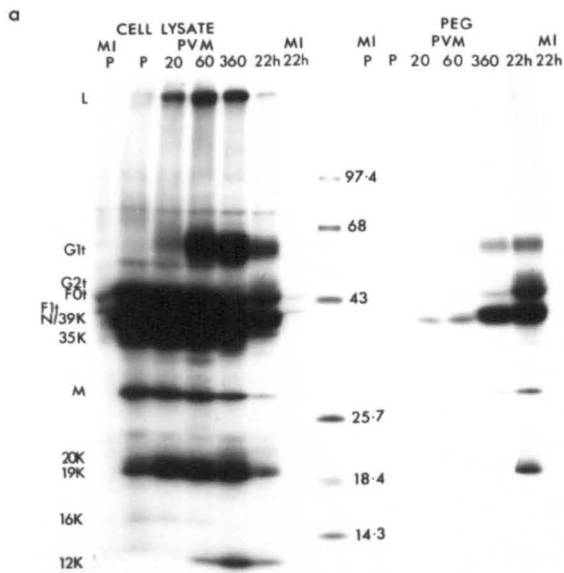
to tell if it was modified to give one of the other polypeptides or if it was simply degraded. No F0 to F1 change was observed only F1 being detected after the pulse period. This could mean that no such change actually occurs and that F1 is synthesized as such *in vivo*, that the change occurs more rapidly than the pulse period could detect or that cleavage occurred *in vitro* during the sample processing. In addition to the changes involving G1 and G2 described in Section 3.3.4 the 12K polypeptide was observed not to be a primary gene product appearing only after about 60 minutes of the chase period. The precursor of this polypeptide was not clear. The absence of F0 at earlier times after the pulse argues against this polypeptide representing F2 whilst its relatively early appearance argues against it being related to the G polypeptides. The size of the polypeptide would be more consistent with it being derived from cleavage of G1 than from cleavage of F0. It is shown later however (Section 3.3.26) that the 12K polypeptide did not share tryptic peptides with G1 and it was therefore not derived from G1.

3.3.21 Pulse chase experiment carried out in the presence of tunicamycin

Two exposures of a gel showing the results of a pulse chase experiment carried out in the presence of tunicamycin are shown in Figure 45. The pattern was similar to that observed in the pulse chase experiment carried out in the absence of tunicamycin described above. The G1t and G2t

Figure 45 Pulse chase experiment carried out in the presence of tunicamycin using immunoprecipitation with anti-PVM serum

PVM infected and mock infected cells were incubated in the presence of tunicamycin (2.5 μ g per ml) for 48 hours at 33°C before labelling for 10 minutes with [³⁵S]-methionine (400 μ Ci per ml) and chasing for various lengths of time in the absence of labelled methionine as described in Section 2.5.13. The pulse labelling and chase periods were carried out in the presence of tunicamycin. The cell lysates and PEG precipitates of the supernatants were immunoprecipitated with anti-PVM serum and analysed by SDS-PAGE using a 6-15 per cent gradient gel. Two fluorographs showing different exposures of the gel are shown (a and b). The PVM specific polypeptides are labelled on the left and the M_r s of the marker polypeptides ($\times 10^{-3}$) are indicated in the centre. Samples immunoprecipitated from mock infected cells are indicated by MI above the track all other tracks representing polypeptides immunoprecipitated from PVM infected cells. The length of the chase period is indicated above each track in minutes except for the 22 hour chase period (22h). P indicates that the samples were harvested immediately following the pulse label. Polypeptides immunoprecipitated from cell lysates are on the left of the marker track (labelled cell lysate) and polypeptides immunoprecipitated from PEG precipitates of cell supernatants are shown on the right (labelled PEG). The shorter exposure (b) shows immunoprecipitates of cell lysates only.



polypeptides were observed in place of the G1 and G2 polypeptides and there were differences in the region of the F1 polypeptide. A band occurring in a similar position to F1 was observed to become reduced in intensity after the longer chase periods and an additional polypeptide migrating slightly slower than N became apparent. This is more apparent in the short exposure shown in Figure 45b. Although some of the polypeptide with a mobility similar to F1, (designated F0t), was released into the supernatant this was matched by a greater release of the faster mobility polypeptide F1t so it would appear that F1t was derived from F0t no other suitable precursor being identified. These bands therefore may represent the unglycosylated forms of F0 and F1, the latter being derived from the former during the chase period. The conversion of F0 to F1 was not observed in the experiments described in Section 3.3.20 so the cleavage of F0t must occur more slowly than that of the native polypeptide F0. An alternative explanation would be that F1 was the initial product produced but that this was sensitive to protease activity in the presence of tunicamycin. If a modification of F1 rendering it protease sensitive did occur it was not reflected by any change in mobility.

3.3.22 Pulse chase experiment using [³H]-glucosamine labelling

A pulse chase experiment was carried out using [³H]-glucosamine labelling to find if the F like polypeptide could be observed in this manner. The gel obtained is shown

Figure 46 Pulse chase experiment carried out using
[³H]-glucosamine labelling

PVM and RS virus infected cells were incubated for 48 hours at 33°C and then labelled for 10 minutes with [³H]-glucosamine (400μCi per ml) and incubated in medium without [³H]-glucosamine for 3 hours as described in Section 2.5.13. PVM specific polypeptides are labelled on the left whilst RS virus specific polypeptides are labelled on the right. Molecular weights ($\times 10^{-3}$) of the marker proteins are shown to the right of the marker track.

Lysate-	Polypeptides immunoprecipitated from cell lysates.
PEG-	Polypeptides immunoprecipitated from PEG precipitates of cell supernatants.
anti-PVM-	PVM polypeptides immunoprecipitated using anti-PVM serum.
43.1-	RS virus infected polypeptides immunoprecipitated using the anti-F monoclonal antibody 43.1.
111.2a-	RS virus polypeptides immunoprecipitated using the anti G monoclonal antibody 111.2a.
P-	Polypeptides immunoprecipitated from pulse labelled samples.
C-	Polypeptides immunoprecipitated from samples pulse labelled and then chased for 3 hours in the absence of label.

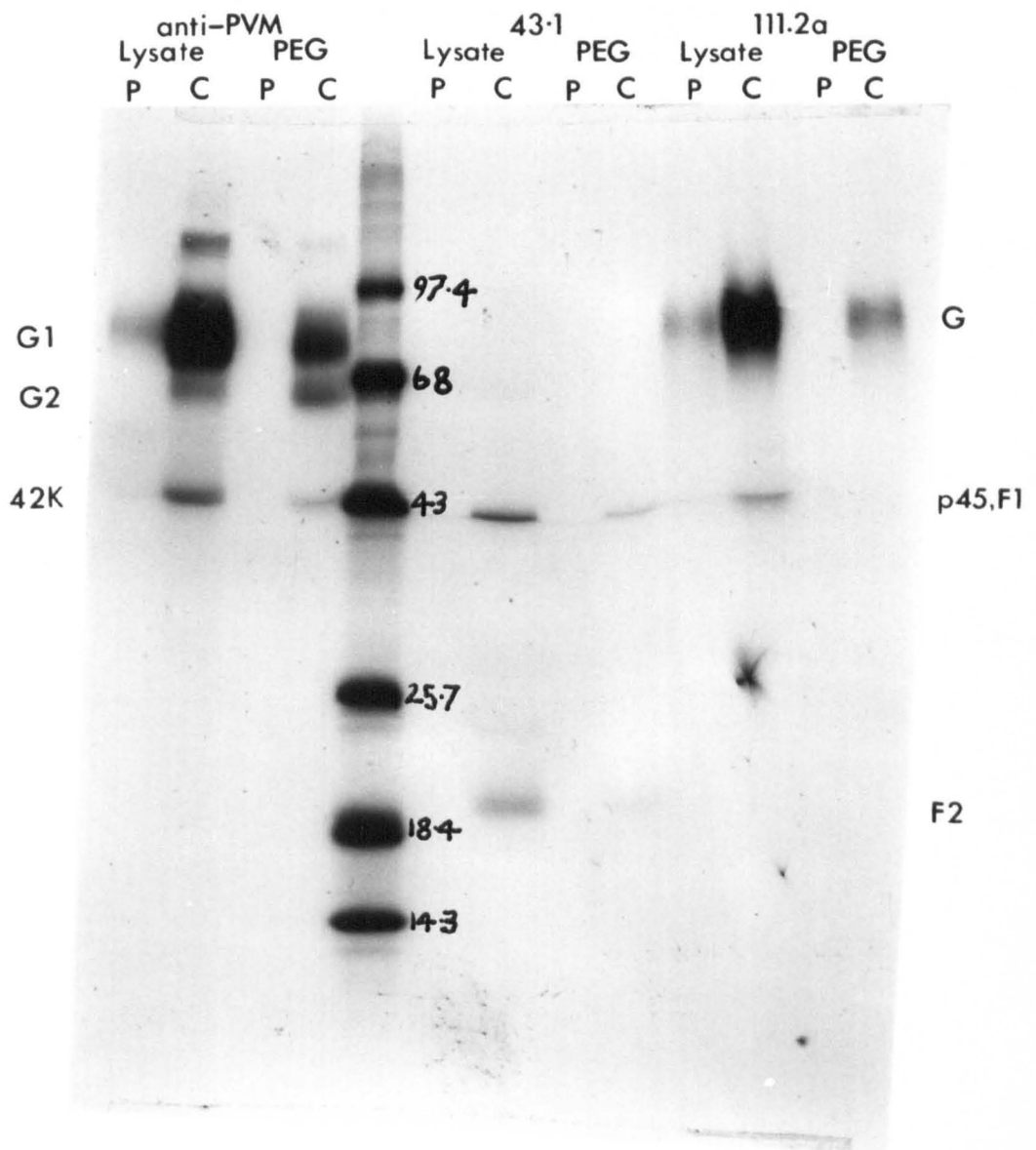
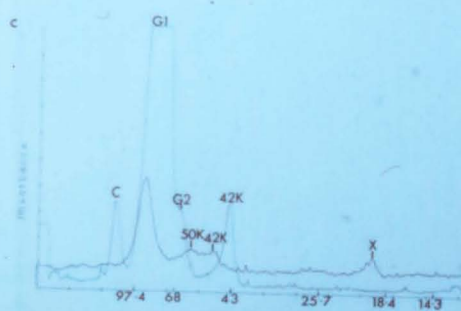
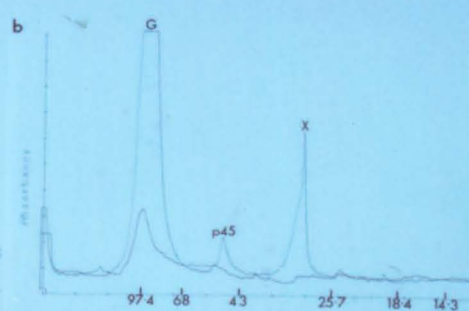
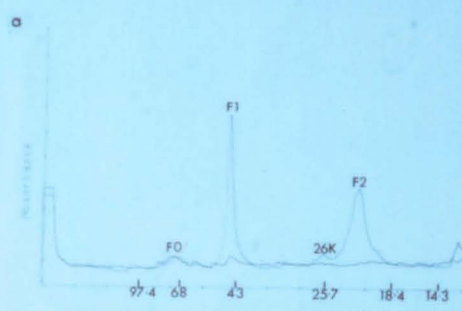


Figure 47 Pulse chase experiment using [^3H]-glucosamine,
densitometer traces.

Tracks from the gel shown in Figure 46 were scanned with a densitometer in order to show some of the weaker bands more clearly.

- (a) Immunoprecipitates of the RS virus F protein related polypeptides occurring after a pulse labelling (black) and pulse labelling followed by a chase period (green).
- (b) Immunoprecipitates of the RS virus G protein related polypeptides occurring in RS virus infected cell lysates after a pulse labelling (black) and pulse labelling followed by a chase period (green).
- (c) Immunoprecipitates of the PVM glycoproteins occurring in cell lysates of PVM infected cells after a pulse labelling (black) and pulse labelling followed by a chase period (green).



in Figure 46 and since the labelling of the polypeptides during the pulse period was poor densitometer traces of the gel tracks are shown in Figure 47. The equivalent peaks did not exactly overlap probably due to distortion on the gel. There was a considerable increase in the degree of labelling observed after the chase period compared to that observed immediately following the pulse. This may have been due to relatively low levels of glucosamine in intracellular pools compared to amino acids which was made more noticeable by the absence of a high level of cold glucosamine in the medium used to wash the cells. Additional glucosamine was not added to the medium since this was reported to be an inhibitor of glycosylation at high concentrations and may have altered the results (Klenk et al., 1972; Koch et al., 1979; Schwartz & Klenk, 1974). The pulse labelled samples were weakly labelled, no labelling at all being observed in the supernatants of pulse labelled samples as would be expected. RS virus infected cell samples immunoprecipitated with anti-F and anti-G monoclonal antibodies are also shown in Figures 46 and 47. The RS virus F polypeptide related bands showed similar relationships to those described by Fernie et al. (1985) and Gruber & Levine (1983). Bands of low intensity were observed in the pulse labelled sample that corresponded to F0 and F1. The chase sample revealed an F0 band of similar intensity to that observed after the chase period probably due to continuing incorporation of label into this polypeptide during the chase period. Strong F1 and F2 bands were observed in the chase sample in

agreement with the results of the authors mentioned above who showed that F0 was converted to F1 and F2 during the chase period. The high mobility peaks observed in Figure 47a corresponded to marks on the autoradiograph and did not represent genuine polypeptide peaks. The supernatant did not contain detectable levels of F related polypeptides after the pulse period but RS F1 and F2 polypeptides appeared in the supernatant after the chase period. Two RS G related polypeptides were observed. The peak labelled X in Figure 47b was due to an artefact on the fluorograph not a virus specific band. In contrast to the results of Fernie *et al.* (1985) and Gruber & Levine (1985b) but in agreement of those of Routledge *et al.* (1986) there was no obvious precursor product relationship between the p45 polypeptide and G. This might be expected since [³H]-glucosamine would be incorporated directly into G1 if it was derived from a less highly glycosylated precursor.

The polypeptides observed in the cell lysates of PVM infected cells are shown in Figure 47c. The peak labelled X was due to a mark on the fluorograph. Three virus specific peaks could be identified on the track of pulse labelled samples, the smaller two not being well resolved on the densitometer trace. The 50K polypeptide may be F0. The 42K polypeptide band may represent F1 and/or the 42K polypeptide with a similar mobility that is related to G1 and G2. After the chase period the polypeptide marked C which was a cellular polypeptide immunoprecipitated by the anti-PVM serum, became apparent as did G2 (not fully resolved in

Figure 47d but clearer in Figure 46), whilst the 50K polypeptide disappeared. The 50K polypeptide could have been a precursor to G1, the cellular polypeptide or a glycosylated F1 polypeptide that would co-migrate with the 42K polypeptide. No detectable PVM specific polypeptides were released into the supernatant during the pulse period, whilst the pattern observed following the chase period was similar to that observed in the cell lysate samples obtained after the chase period. The G2 polypeptide was relatively more abundant in the supernatant as was usually observed.

3.3.23 Association of G1, G2 and F1 with a particulate fraction in PVM infected cell supernatants.

Figure 48 shows that when the supernatant of PVM infected cell cultures was pelleted at 100,000g for 60 minutes the pellet and supernatant showed different polypeptide profiles following immunoprecipitation with anti-PVM serum. The supernatant fractions contained a high level of background host cell polypeptides that were virtually absent from the pellet. The supernatant fractions also contained PVM G1, N and M polypeptides and perhaps a low level of G2. The polypeptides observed were probably soluble protein released from disrupted cells or virions. The experiment was originally performed in an attempt to demonstrate the presence or absence of a soluble non-virion glycoprotein, but the high levels of several polypeptides in the supernatant suggested that widespread disruption of cells or virions had occurred so it was not possible to tell

Figure 48 Polypeptides associated with soluble and particulate fractions of PVM infected cell supernatants

Clarified supernatant of PVM infected BS-C-1 and BHK-21 cells and mock infected BS-C1 cells was pelleted at 100,000g for 60 minutes and the pellet and supernatant fractions immunoprecipitated with anti-PVM serum. The immunoprecipitates were analysed by SDS-PAGE using a 6-15 per cent gradient gel. A fluorograph of the gel is shown in the figure. The PVM specific polypeptides are labelled on the left.

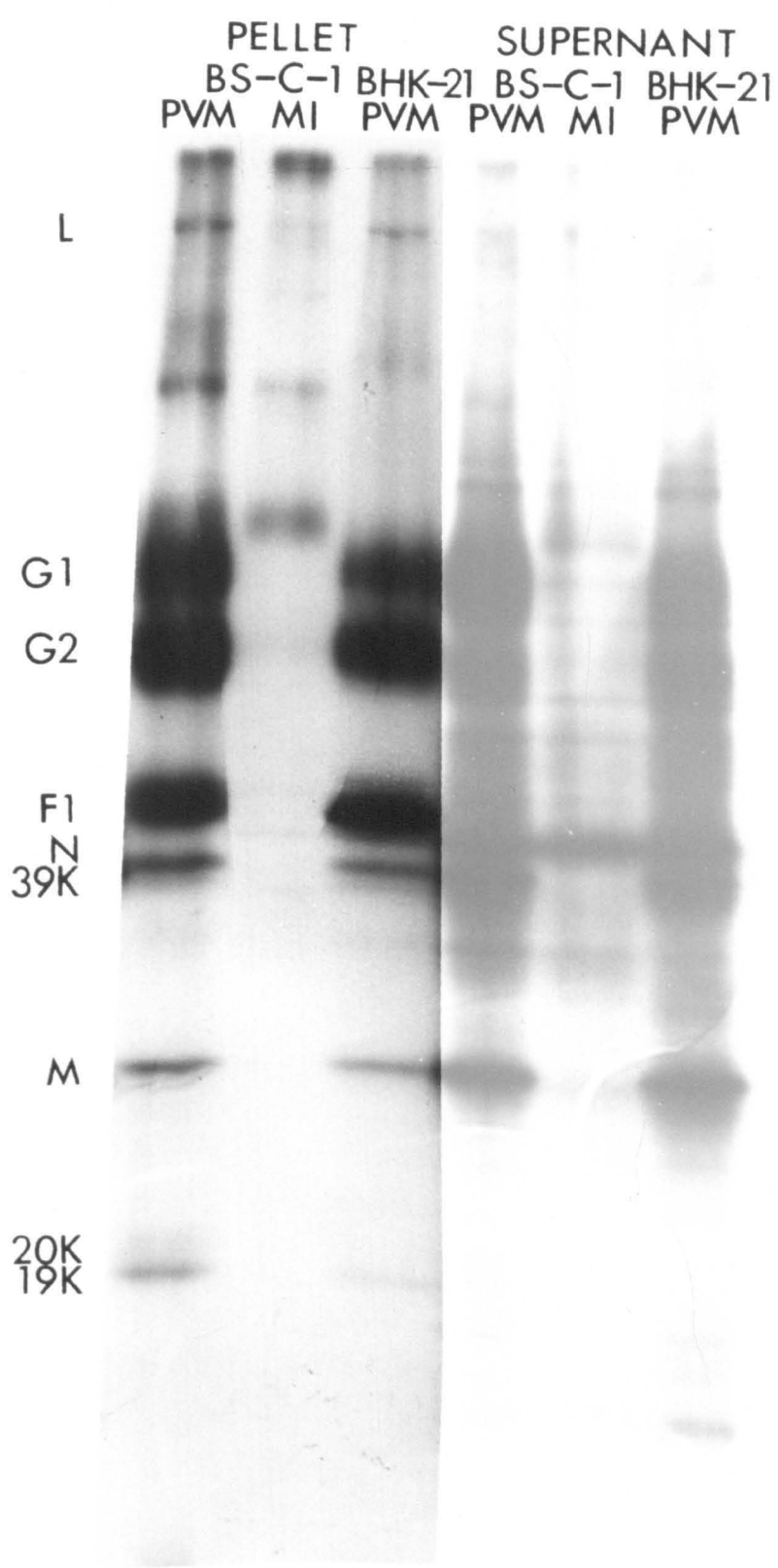
Pellet Immunoprecipitates of the pellet fraction.
Supernatant Immunoprecipitates of the supernatant fraction.

BS-C-1 Immunoprecipitates of polypeptides in samples derived from the supernatants of BS-C-1 cells.

BHK-21 Immunoprecipitates of polypeptides in samples derived from the supernatant of BHK-21 cells.

PVM Immunoprecipitates of polypeptides in samples from the supernatants of PVM infected cells.

MI Immunoprecipitates of polypeptides in samples from the supernatants of mock infected cells.



if any particular protein was secreted. The pellet appeared to be relatively enriched in G1, G2 and F1 suggesting that these polypeptides may have been associated in some common structure, and also contained the other virus structural polypeptides. Insufficient material precluded assaying the fractions for infectivity so it is not clear if the pellet did in fact represent virions or disrupted membranes.

3.3.24 The effect of Glycopeptidase F on PVM and RS virus glycoproteins

Glycopeptidase F cleaves the bond between asparagine residues and the carbohydrate chain of N-linked glycoproteins. This enzyme was used to demonstrate directly the effect on mobility of removing N-linked carbohydrate from the G1, G2, G1t, G2t, F1 and F1t glycoproteins of PVM and the F1 and F2 glycoproteins of RS virus. The PVM glycoproteins were labelled with either [^{35}S]-methionine (Figure 49) or [^3H]-glucosamine (Figure 50). The RS virus polypeptides were labelled with [^3H]-glucosamine (Figure 50). It can be seen that the RS virus F1 and F2 glycoproteins were deglycosylated by this enzyme under the conditions used (Figure 50). Unfortunately insufficient label was incorporated into the RS virus G polypeptide in this experiment to study the effect of the enzyme on this polypeptide. The PVM G1 polypeptide was observed to show an increase in mobility following digestion with glycopeptidase F, the polypeptide co-migrating with the G1t polypeptide (Figure 49) as would be expected if G1t represented the G1

**Figure 49 The effect of glycopeptidase F on PVM
glycoproteins**

PVM infected cells were labelled 48 hours after infection, in the presence or absence of tunicamycin (2.5 μ g per ml) for 24 hours with [35 S]-methionine (100 μ Ci per ml). The polypeptides were immunoprecipitated with anti-PVM serum and separated by SDS-PAGE using a 6-15 per cent gradient gel. The PVM G1, G2, G1t, G2t and F1 polypeptides were excised from the gel using a fluorograph as a template. The polypeptides were eluted and concentrated as described in Section 2.5.25. samples of each polypeptide equivalent to one band on the gel were digested with glycopeptidase F or incubated in buffer without enzyme as described in Section 2.5.25. The samples were boiled with sample buffer and the polypeptides analysed on a 6-15 per cent gradient gel. The figure shows a fluorograph of this gel. The polypeptide indicated above each pair of tracks was either digested with glycopeptidase F (N) or undigested (U). The M_r s of the markers are indicated to the right of the marker track.

G1		G1t		G2		G2t		F1	
U	N	U	N	U	N	U	N	U	N

200

97.4

68

43

25.7

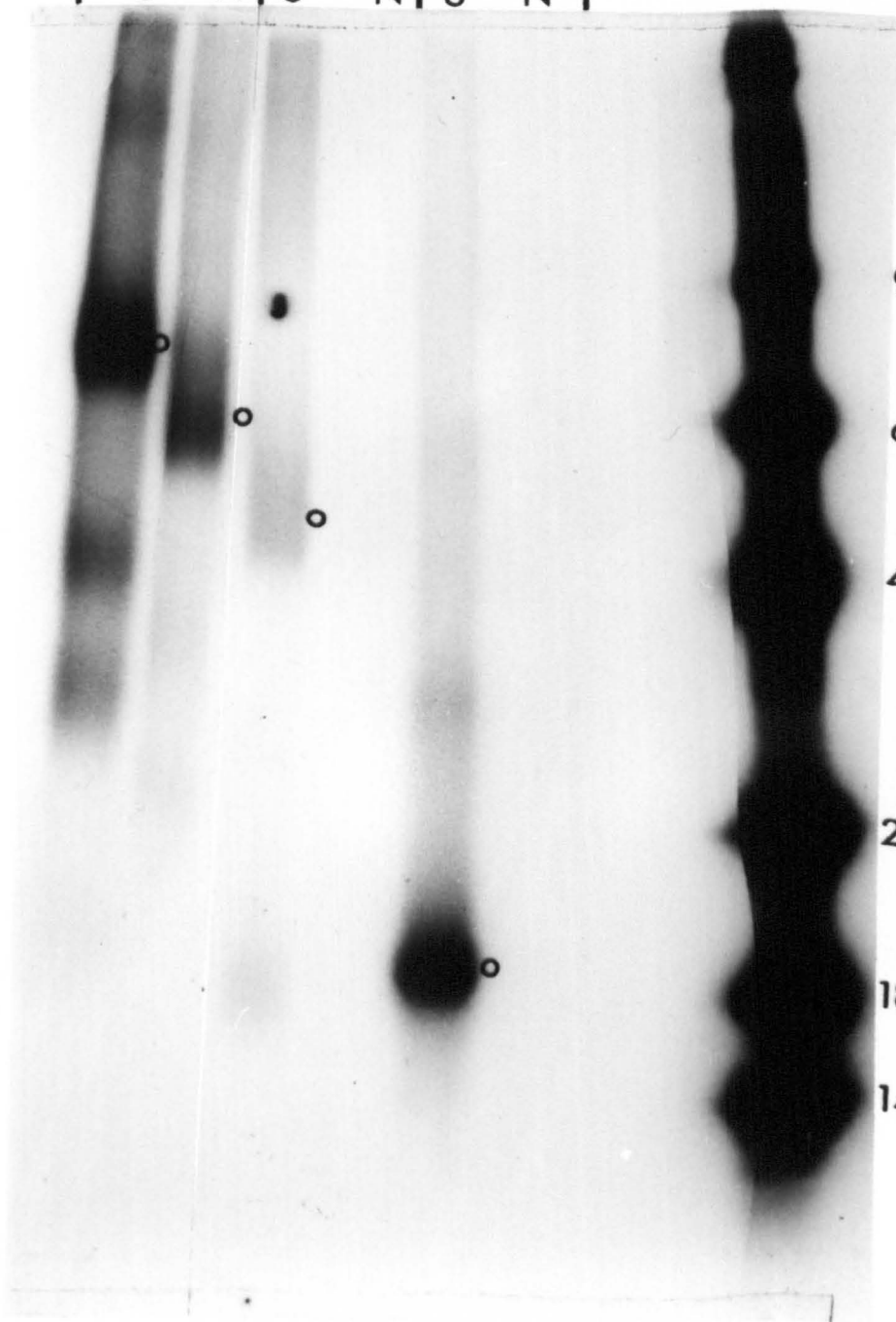
18.4

14.3

Figure 50 Digestion of [³H]-glucosamine labelled PVM and RS virus polypeptides with glycopeptidase F

PVM and RS virus infected cells were labelled 48 hours after infection, in the presence or absence of tunicamycin (2.5 μ g per ml) with [³H]-glucosamine (100 μ Ci per ml) for 24 hours. The labelled polypeptides were immunoprecipitated with homologous antisera and separated by SDS-PAGE using a 6-15 per cent gradient gel. The G1 polypeptide of PVM and the F1 and F2 polypeptides of RS virus were excised from the gel, digested or mock treated and analysed on a second gel as described in Section 2.5.25. A fluorograph of the gel is shown in the figure. The polypeptide indicated above each pair of tracks was digested with glycopeptidase F (N) or undigested (U).

PVM		RS virus	
G1		F1	F2
U	N	U	N



97.4

68

43

25.7

18.4

14.3

polypeptide without its N-linked carbohydrate. A similar result was obtained by digestion of the [³H]-glucosamine labelled G1 polypeptide (Figure 50). The other PVM glycoproteins were not sufficiently well labelled to study the effect of glycopeptidase F on the [³H]-glucosamine labelled polypeptides. Digestion of G2 gave a band with a mobility similar to G2t (Figure 49). The G1t and G2t polypeptides did not show any mobility change after digestion as would be expected since these polypeptides should not have possessed N-linked carbohydrate. The other higher mobility polypeptides observed in each track were probably breakdown products of the major bands described above. These results provided further evidence that a band with the mobility of G1t represented G1 without its N-linked oligosaccharides and a band with the mobility of G2t represented G2 without its N-linked oligosaccharides. Digestion of F1 did not result in a mobility change which does not support the result obtained using tunicamycin when a polypeptide of increased mobility was observed. The reason for this is not known, the enzyme may have failed to act on the F1 protein or the presence of F1t rather than F1 in tunicamycin treated PVM infected cells may have been due to some effect other than failure to add N-linked oligosaccharides.

3.3.25 Digestion of PVM and RS virus glycoproteins with

Endo- α -N-acetylgalactosaminidase

Endo- α -N-acetylgalactosaminidase cleaves the bond

**Figure 51 Digestion of PVM glycoproteins with
Endo- α -N-acetylgalactosaminidase**

PVM infected cells were labelled, in the presence of tunicamycin (2.5 μ g per ml), with [35 S]-methionine as described in the legend to Figure 49. The PVM G1t and G2t polypeptides were excised from a gel of polypeptides immunoprecipitated with anti-PVM serum, eluted and concentrated as described in Section 2.5.25. All of the samples of the polypeptides were digested with a mixture of exoglycosidases. In addition one sample of each polypeptide was digested with Endo- α -N-acetylgalactosaminidase and one sample with both Endo- α -N-acetylgalactosaminidase and glycopeptidase F (Section 2.5.25.). The digests were analysed by SDS-PAGE using a 6-15 per cent gradient gel. A fluorograph of this gel is shown in the figure. The polypeptide indicated above each track was digested with the exoglycosidases only (E), with exoglycosidases and Endo- α -N-acetylgalactosaminidase (O), or with exoglycosidases, Endo- α -N-acetylgalactosaminidase and glycopeptidase F (N+O). The M_r s of the markers ($\times 10^{-3}$) are indicated to the right of the marker track.

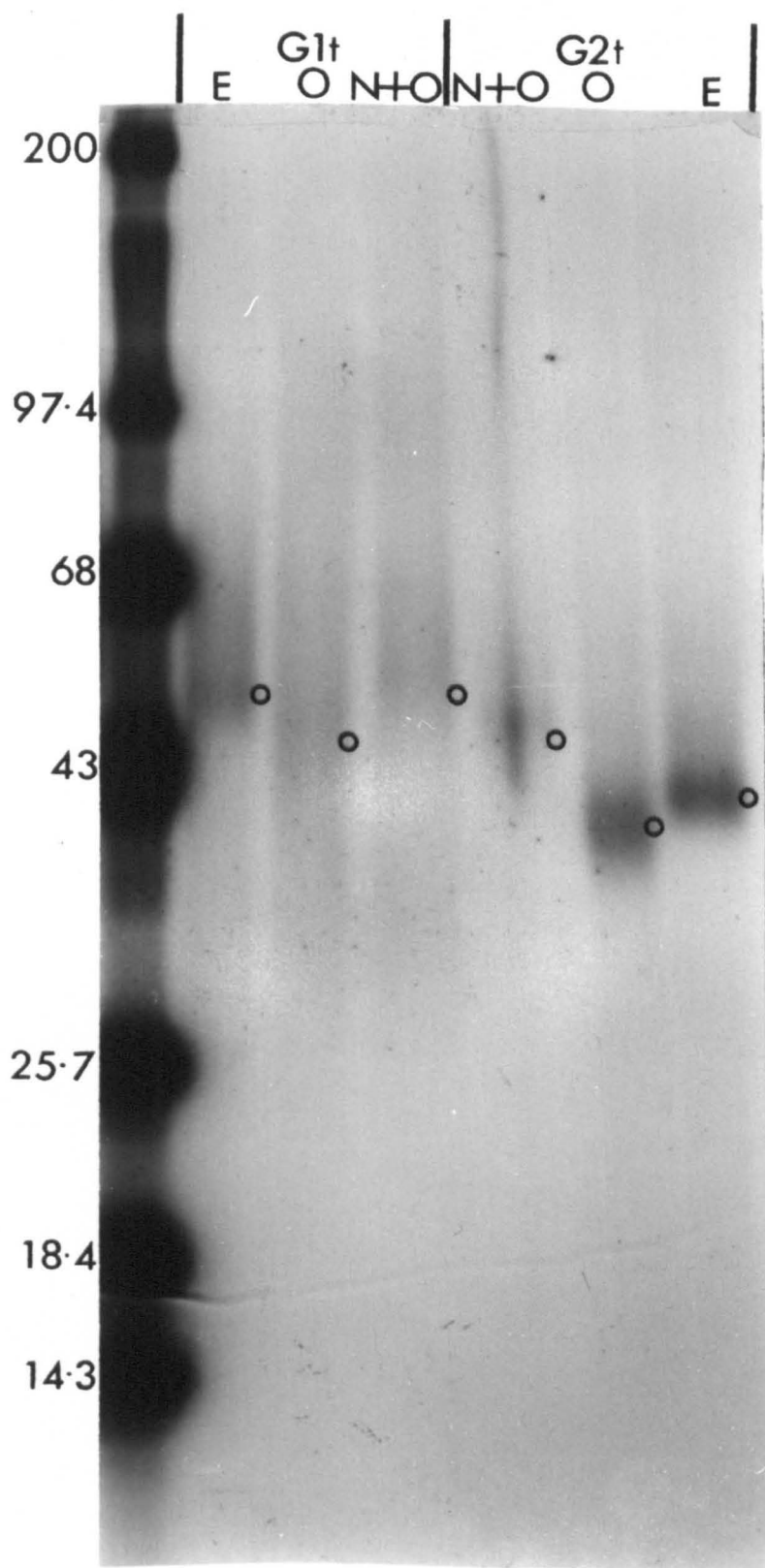


Figure 52 Digestion of PVM polypeptides labelled with [³H]-glucosamine with Endo- α -N-acetylgalactosaminidase.

PVM polypeptides were labelled 48 hours after infection with [³H]-glucosamine (100 μ Ci per ml) for 24 hours. The polypeptides were immunoprecipitated with anti-PVM serum and separated by SDS-PAGE using a 6-15 per cent gradient gel. The bands were excised, the polypeptides eluted and samples digested with various combinations of enzymes as described in the legend to Figure 51. The samples were analysed on a 6-15 per cent gradient gel a fluorograph of which is shown in the figure. The polypeptide indicated above each group of three tracks was digested with exoglycosidases only, (E), exoglycosidases and Endo- α -N-acetylgalactosaminidase (O) or exoglycosidases, Endo- α -N-acetylgalactosaminidase and glycopeptidase F (N+O). The M_r s of the markers ($\times 10^{-3}$) are shown on the left of the marker track.

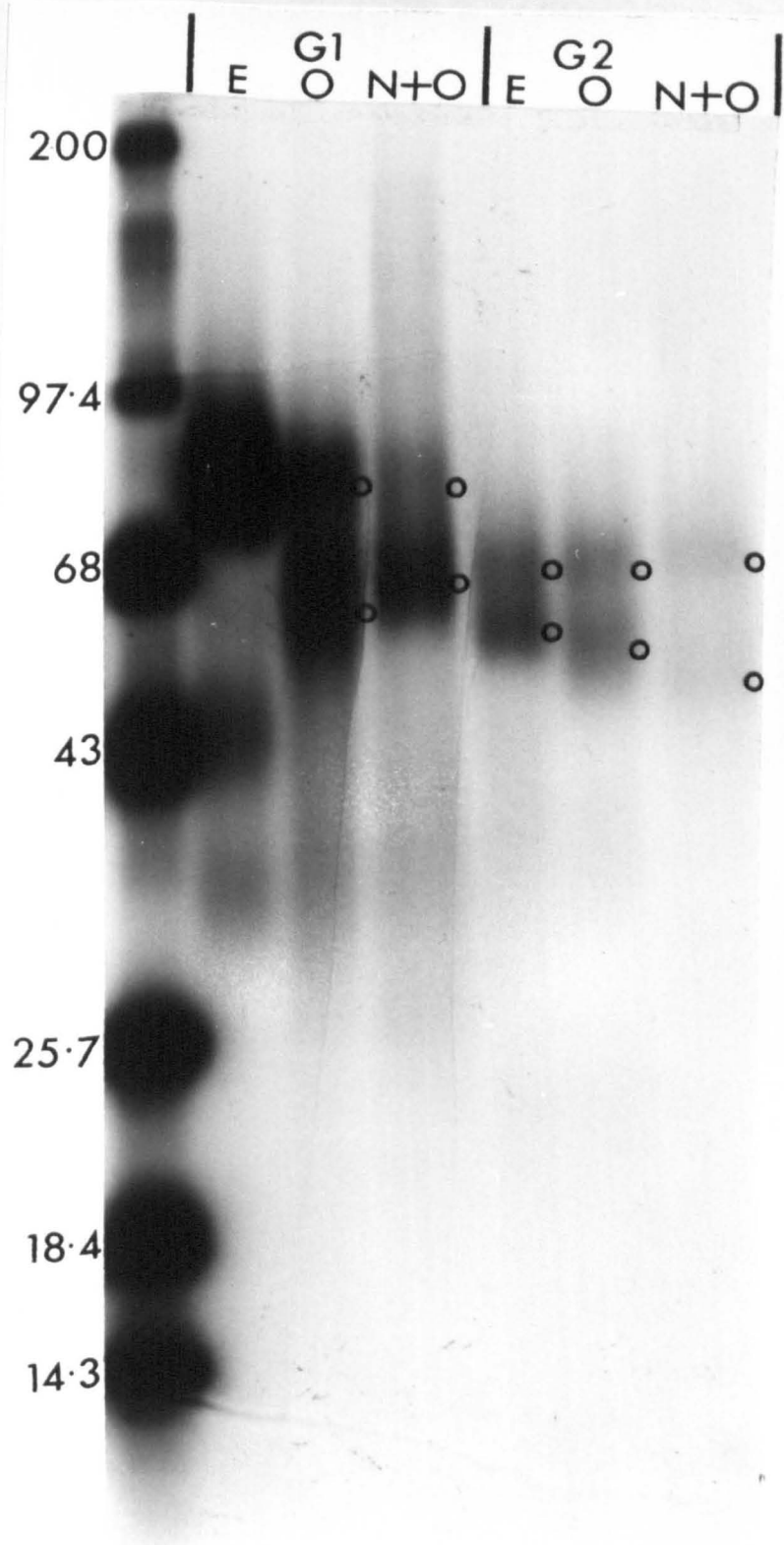
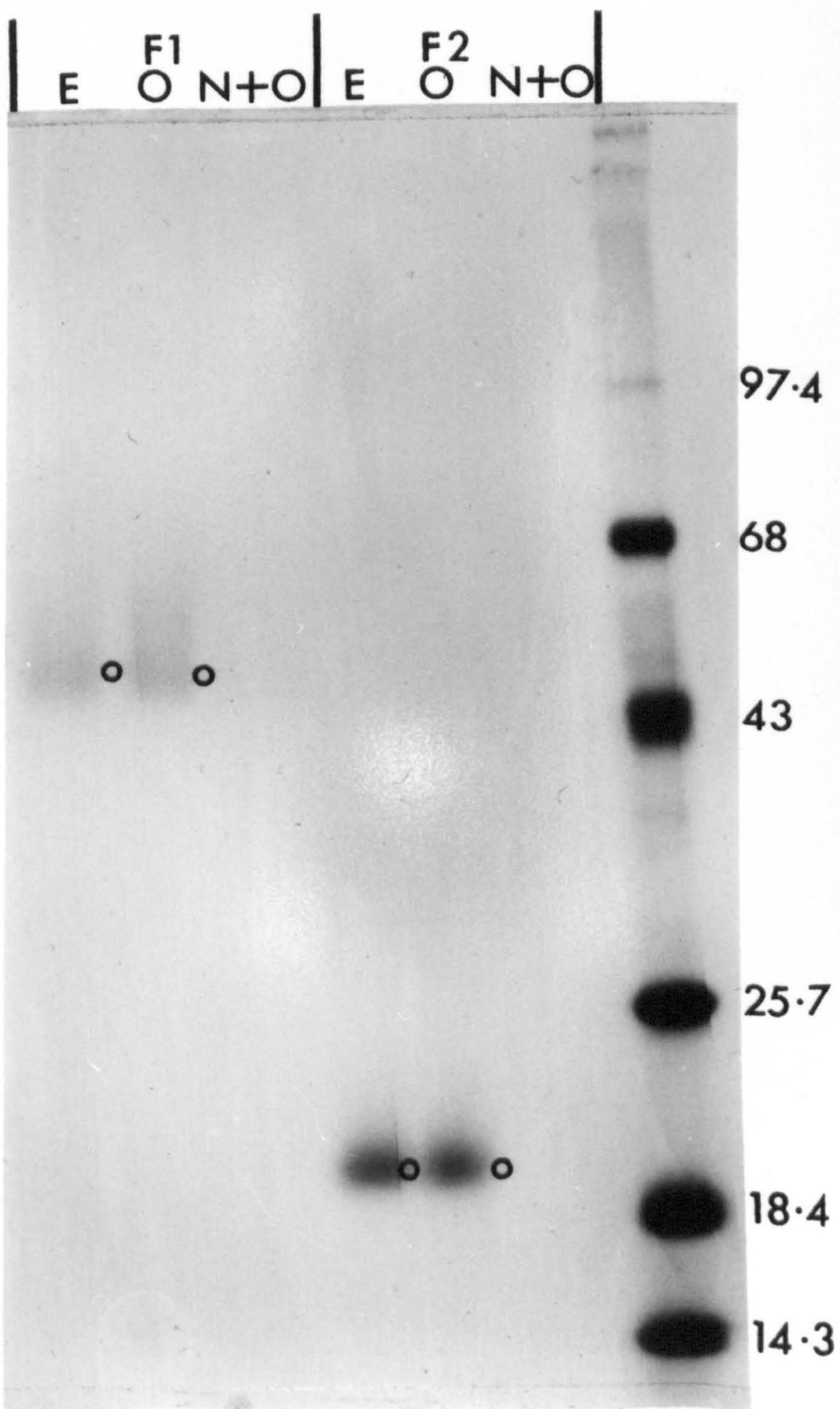


Figure 53 Digestion of RS virus polypeptides labelled with [³H]-glucosamine with Endo- α -N-acetylgalactosaminidase.

RS virus polypeptides were labelled 48 hours after infection with [³H]-glucosamine, (100 μ Ci per ml) for 24 hours. The polypeptides were immunoprecipitated with anti-RS virus serum and separated by SDS-PAGE using a 6-15 per cent gradient gel. The bands were excised, the polypeptides eluted and samples digested with various combinations of enzymes as described in the legend to Figure 51. The samples were analysed on a 6-15 per cent gradient gel a fluorograph of which is shown in the figure. The polypeptide indicated above each group of three tracks was digested with exoglycosidases only, (E), exoglycosidases and Endo- α -N-acetylgalactosaminidase (O) or exoglycosidases, Endo- α -N-acetylgalactosaminidase and glycopeptidase F (N+O). The M_r s of the markers ($\times 10^{-3}$) are shown on the right of the marker track.



between the core disaccharide Gal(β 1-3)GalNAc and serine or threonine residues of O-glycans. The enzyme does not function if the disaccharide is substituted by other sugars (Endo & Kobata, 1976). Due to this latter observation and ignorance of the nature of any O-linked oligosaccharides that might exist in the PVM glycoproteins digestion with this enzyme was carried out in the presence of exoglycosidases to remove oligosaccharides from the core disaccharide. The exoglycosidases used were neuraminidase from *Clostridium perfringens* and a mixture of enzymes from *Turbo cornutus*. These enzymes alone were also used to digest the polypeptides as a control so that any mobility change could be associated with the action of Endo- α -N-acetylgalactosaminidase. The results of digestion of PVM glycoproteins labelled with [35 S]-methionine or [3 H]-glucosamine are shown in Figures 51 and 52 respectively. The results of digestion of RS virus polypeptides labelled with [3 H]-glucosamine with these enzymes are shown in Figure 53. In each Figure the result of digestion with both these enzymes and Glycopeptidase F is also shown. The RS G protein did not label adequately to be detectable after digestion (considerable losses of label occurred during the preparation of the proteins for digestion, probably due to non-specific binding of the glycoproteins to dialysis tubing or leakage of the dialysis tubing). The RS virus F1 and F2 glycoproteins did not show any specific changes in mobility due to treatment with Endo- α -N-acetylgalactosaminidase (Figure 53). This was

expected since these polypeptides are known to have only N-linked oligosaccharides (e.g. as was shown in Figure 50 and by Gruber & Levine 1983). When glycopeptidase F was included amongst the enzymes used to digest the RS virus F1 and F2 polypeptides no band was observed as expected for the reason mentioned above.

The [^{35}S]-methionine labelled G1 and G2 glycoproteins of PVM digested with Endo- α -N-acetylgalactosaminidase are not shown since no digestion was observed for unknown reasons since the [^3H]-glucosamine labelled G1 and G2 polypeptides were digested under identical conditions. Figure 51 shows G1t and G2t digested with exoglycosidases, exoglycosidases and Endo- α -N-acetylgalactosaminidase or these enzymes plus glycopeptidase F. The mobilities of G1t and G2t suggest that they were digested to some extent by the exoglycosidases alone. In the case of G1t a similar mobility was observed for the protein digested with all the enzymes (tracks labelled N+O) although for G2t there is no evidence of even exoglycosidase activity when all the enzymes were used in combination. The G1t and G2t polypeptides digested with exoglycosidases and Endo- α -N-acetylgalactosaminidase (tracks labelled O) showed evidence of digestion by Endo- α -N-acetylgalactosaminidase. Although it is possible that the increased mobility was due to contaminating protease or glycosidase activities in the enzyme preparation the results suggest that there were some O-linked oligosaccharides on the G1t and G2t polypeptides. [^3H]-glucosamine labelling of G1t and G2t did not give

adequate label incorporation to carry out glycosidase digestions so it is not known if the forms of G1t and G2t observed following digestion with Endo- α -N-acetylgalactosaminidase represent the fully deglycosylated forms of the G1 and G2 proteins. The failure to detect a similar mobility change when glycopeptidase F was included in the digestion mixture suggests that the compromise conditions used inhibited the action of Endo- α -N-acetylgalactosaminidase.

Figure 52 shows that the G1 and G2 glycoproteins could also be digested with Endo- α -N-acetylgalactosaminidase. Some undigested polypeptide remained in each case. The mobility of G1 appeared to have been unaffected by the exoglycosidase treatment judging by comparison of M_r estimates with those of undigested G1 protein. There was however an increase in the mobility of G2 when it was digested with exoglycosidases. The mobilities of G1 and G2 when digested with all the enzymes (tracks labelled N+0) were similar to those observed with the [35 S]-methionine labelled glycoproteins digested in the same way. Since there was no evidence of the action of Endo- α -N-acetylgalactosaminidase on the [35 S]-methionine labelled polypeptides the mobility change in G1 and G2 when digested with all the enzymes was probably due to the action of glycopeptidase F. The failure of Endo- α -N-acetylgalactosaminidase to digest the proteins in combination with glycopeptidase F would be consistent with the results observed for digestion of G1t and G2t described

above. It can be seen that digestion of both G1 and G2 by Endo- α -N-acetylgalactosaminidase occurred (compare tracks labelled E and O). Taken together the results suggest that the PVM G1 and G2 glycoproteins have O-linked oligosaccharides with G1 probably having a larger extent of O-linked glycosylation than G2.

Calculation of the amounts of each type of carbohydrate was complicated by several factors. The efficiency of the action of Endo- α -N-acetylgalactosaminidase is not known and the M_r s of the carbohydrate components of the glycoproteins calculated from glycoprotein mobilities are not valid (but useful in a descriptive manner). In addition the exoglycosidases removed oligosaccharides from both O-linked and N-linked chains so for G2 where both types of oligosaccharides were present, a mobility change due to the action of exoglycosidases could not be apportioned between the two types of oligosaccharides. A final caveat to the validity of the calculations is that there were not any undigested glycoproteins on these gels so calculation of the amount of carbohydrate removed by the exoglycosidases was less accurate than calculation of removal of N-linked carbohydrate and O-linked disaccharides. The purpose of the calculations was to enable rough estimates of the contributions of the N-linked and O-linked oligosaccharides to the glycoprotein M_r s to be obtained and to estimate the sizes of the unglycosylated forms of the polypeptides. The amount of N-linked carbohydrate (represented by the M_r of

Table 14 Carbohydrate of the G related glycoproteins

Protein (RMM)	Protein M _r after digestion with			Size ¹ due to N-linked	Size ¹ due to O-linked
	N ²	O ²	E ²	Carbohydrate	Carbohydrate
G1 (76.2)	58.2			18.0	
(80.3)		61.5	80.3		18.8 (0.0)
G2 (60.3)	46.5			13.8	
(65.9)		54.9			3.4-11.0
(64.9)		57.3			(7.6)
G1t (58.2)	58.2			0.0	
(62.8)		46.2	52.2		16.6 (10.6)
G2t (46.5)	46.5			0.0	
(49.4)		40.9	44.0		8.5 (5.4)

1) The size is the calculated molecular weight of protein causing a similar change in mobility to the removal of the carbohydrate. In the case of G2 an interval of sizes for the O-linked carbohydrate from a minimum based on all the carbohydrate removed by exoglycosidases being N-linked to a maximum based on all such carbohydrate being O-linked is given. The figures in brackets after the size due to O-linked oligosaccharide are the calculated sizes of carbohydrate removed by exoglycosidases.

2) N- Glycopeptidase F

O- Endo- α -N-acetylgalactosaminidase + mixed
exoglycosidases

E- Mixed exoglycosidases

**Table 15 Calculated M_r of G glycoproteins without
N-linked and/or O-linked carbohydrate**

Protein	M_r following removal of :-			
	Nothing	N-linked carbohydrate	O-linked carbohydrate	N- and O- linked carbohydrate
G1	76.4	58.4	57.6	39.6
G2	62.0	48.2	51-58.6 ¹	37.2-44.8 ¹
G1t	60.9	60.9	44.3	44.3
G2t	49.8	49.8	41.3	41.3

1) Lower estimate based on the higher estimate of the amount of O-linked oligosaccharide given in Table 3.3.1. Upper estimate based on the lower estimate of the amount of O-linked carbohydrate given in Table 14.

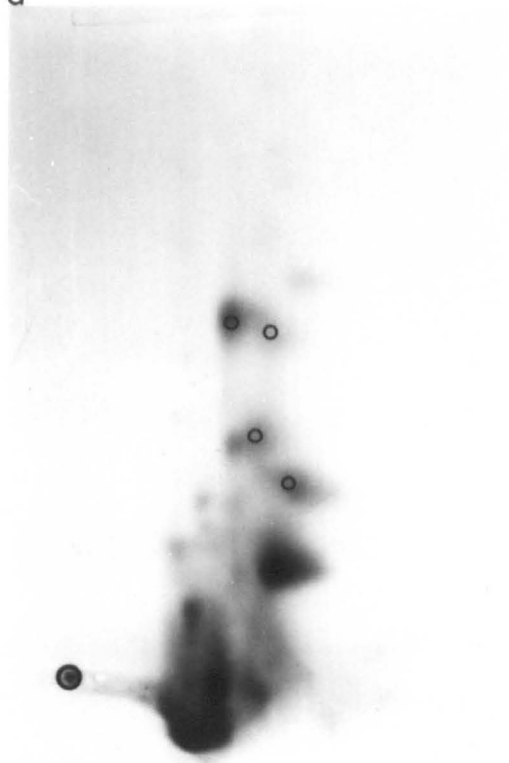
protein giving an equivalent mobility change) was estimated by subtracting the M_r of the protein observed after digestion by glycopeptidase F from that of the undigested polypeptide (Table 14). The amount of O-linked carbohydrate was calculated for G1, G1t and G2t by subtracting the M_r s of the polypeptide observed after digestion by exoglycosidases and Endo- α -N-acetylgalactosaminidase from those of the undigested polypeptides. This was possible because the G1 protein was not digested by exoglycosidases alone and the G1t and G2t polypeptides lacked N-linked oligosaccharides so the exoglycosidases would only be digesting O-linked oligosaccharides. G2 had both O-linked and N-linked oligosaccharides and was digested by the exoglycosidases so the amount of O-linked carbohydrate could not be determined. Limits for the amount of O-linked oligosaccharide were obtained by considering all of the sugars removed by the exoglycosidases to be N-linked (giving a minimum estimate for the amount of O-linked carbohydrate) and by considering all of the carbohydrate removed by exoglycosidases to be O-linked (giving a maximum estimate for the amount of O-linked oligosaccharide). The calculated M_r s of the G related glycoproteins without N-linked, O-linked or N-linked and O-linked oligosaccharides are shown in Table 15. These figures suggest that the difference in size of the G1 and G2 proteins is at least partly due to differences in the extent of O-linked glycosylation and if the action of Endo- α -N-acetylgalactosaminidase on G1t was incomplete there may not be any difference in the polypeptide chain of G1 and

Figure 54 Tryptic peptide maps of G1 and the 12K polypeptide

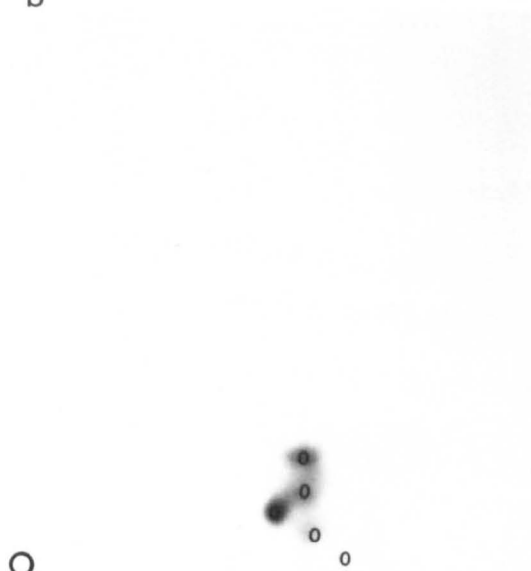
The G1 and 12K polypeptide bands were excised from gels of PVM polypeptides labelled with [^{35}S]-methionine (500 μCi per ml) and 4,5- [^3H]-leucine, (750 μCi per ml) and immunoprecipitated with anti-PVM serum. The polypeptides were digested with trypsin as described in Section 2.5.24 and the peptides analysed by two dimensional thin layer electrophoresis (left to right)/thin layer chromatography (bottom to top). Spots due to G1 peptides are indicated by small circles and spots due to 12K polypeptide peptides are indicated by ovals. Large circles indicate the point of application of the samples.

- (a) Fluorograph of a tryptic peptide map of G1.
- (b) Fluorograph of a tryptic peptide map of the 12K polypeptide.
- (c) Fluorograph of a tryptic peptide map of mixed G1 and 12K polypeptides.

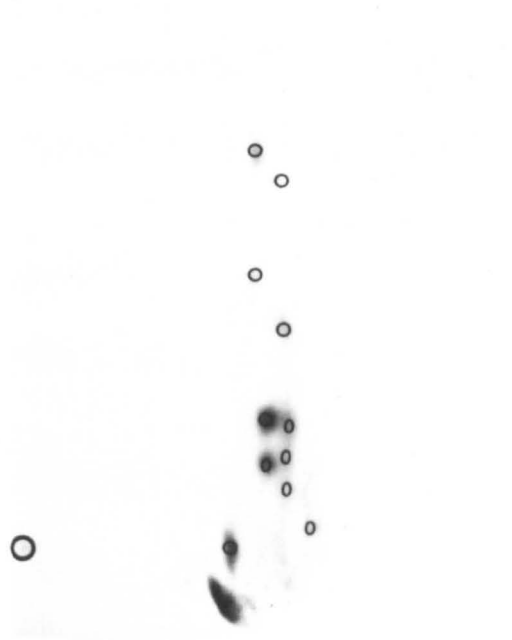
a



b



c



G2.

3.3.26 Tryptic peptide map of the 12K polypeptide

The 12K polypeptide was observed to be expressed on the cell surface (Figure 39) and to appear during chase periods in pulse chase experiments (Figure 44). The intensity of the polypeptide band under non-reduced conditions and failure to detect an F0 polypeptide in pulse chase experiments argued against this polypeptide being related to the F protein. The 12K polypeptide could have been derived from G1 during its change into G2. To investigate this possibility tryptic peptide maps of G1 and the 12K polypeptide were compared. In Figure 54 the G1 peptide spots are indicated by circles and the 12K polypeptides by ovals. It can be seen that the polypeptides did not share common peptides and were therefore not related.

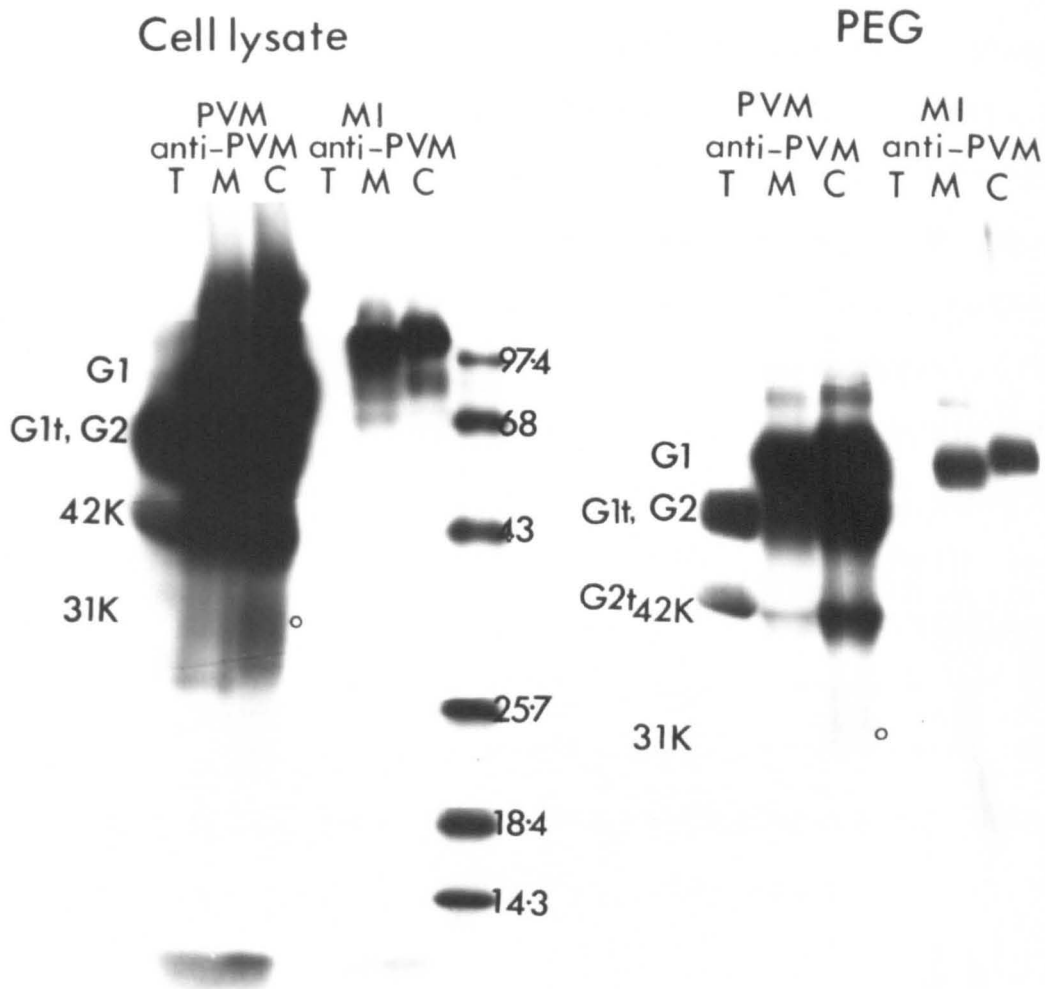
3.3.27 Identification of a 31K glycosylated PVM polypeptide

Figure 55 is a reproduction of Figure 30 and a longer exposure of Figure 29. This figure reveals the presence of a glycosylated 31K polypeptide. This band was observed to exist as a broad smear and was only observed after prolonged exposure of heavily labelled samples. No equivalent polypeptide has been reported in RS virus infected cells labelled with [³H]-glucosamine and immunoprecipitated with anti-RS virus serum. An antiserum raised against a synthetic peptide corresponding to the carboxy-terminus of the RS NS1A

Figure 55 Identification of a 31K PVM glycoprotein

This figure shows a longer exposure of the gel shown in Figure 29 and a reproduction of Figure 30. PVM polypeptides immunoprecipitated from [³H]-glucosamine labelled cell lysates and PEG precipitates of cell supernatants using anti-PVM serum are shown on these fluorographs. The PVM specific polypeptides are labelled on the left and the 31K polypeptide indicated by a circle to the right of the tracks in which it is visible.

- T- Polypeptides immunoprecipitated from samples of cells labelled in the presence of tunicamycin.
- M- Polypeptides labelled in the presence of monensin prior to immunoprecipitation.
- C- Polypeptides labelled in the absence of inhibitors prior to immunoprecipitation
- PVM- Polypeptides immunoprecipitated from samples from PVM infected cells
- MI- Polypeptides immunoprecipitated from samples from mock infected cells.



protein did immunoprecipitate a glycoprotein from [³H]-glucosamine labelled cell lysates which gave a very diffuse band of about 30K when analysed by SDS-PAGE and fluorography (C. Caravokyri, R. Olmsted, personal communications). A similar polypeptide was identified in immunoprecipitates of ³H-glucosamine labelled TRT virus polypeptides, (Section 3.5.8). This polypeptide was reduced in intensity when cells were labelled in the presence of monensin. This effect was probably just due to a reduction in the overall level of protein synthesis in monensin treated cells. This polypeptide was not observed in tunicamycin treated cells suggesting that the oligosaccharides were all N-linked. An equivalent broad band was never observed in [³⁵S]-methionine labelled immunoprecipitates of PVM infected cells. This could mean that no polypeptide was actually associated with this band or simply that the methionine content was low or the quantity of the polypeptide insufficient to be detectable above the background of non-specifically precipitated polypeptides.

3.3.28 Summary

The results presented in this section describe the glycoproteins of PVM, the effect of inhibitors of N-linked and O-linked glycosylation on their synthesis and precursor product relationships observed during pulse chase experiments. The previously reported glycoproteins G1 and G2 were shown to have both N-linked and non-N-linked

oligosaccharides. These polypeptides were also shown to represent different forms of a single glycoprotein, G2 being derived from G1 during the course of pulse chase experiments. The totally deglycosylated forms of the two proteins were not identified so it was not possible to determine if the difference resided in the polypeptide moiety, the carbohydrate moiety or both. The mobility difference between the two forms of the G protein was preserved in the absence of N-linked carbohydrates so these were probably not involved in determining the mobility differences of G1 and G2. The absence of any effect of the presence of α_2 -macroglobulin on the relative intensities of the G1 and G2 bands suggested that endoproteolytic cleavage of G1 during sample preparation or extracellularly during the labelling period was unlikely to account for the presence of G2. If intracellular endoproteolytic cleavage of G1 occurred a small fragment would be produced in addition to G2. This fragment could have been digested or remained intact. The 12K polypeptide was observed to appear during pulse chase experiments at about the same time as G1. The intensity of this band did not correlate with that of G2 suggesting that it was not a product of cleavage of G1. Exoprotease activity would be expected to give a continuous distribution of polypeptide sizes and so is unlikely to account for the apparent modification of G1 to give G2 during pulse chase experiments. An intracellular endoproteolytic cleavage or differences in the non-N-linked oligosaccharides therefore appear to be the most likely

explanations of the relationship between G1 and G2.

In pulse chase experiments no G related polypeptide could be identified until about 60 minutes after labelling with [^{35}S]-methionine. In a pulse chase experiment with [^3H]-glucosamine G1 was observed after the pulse period but this could have been due to incorporation of oligosaccharides into carbohydrate of a polypeptide that had already undergone some processing. The nature of the precursor to G1 is therefore unknown. A small conformational change could change the protein from a form not recognized by antibody to one that is. The PVM G protein may however have a precursor with a lower M_r as is the case with the RS virus G protein. The RS virus G protein has a series of precursors which are believed to represent forms with no O-linked oligosaccharides and varying amounts of N-linked oligosaccharides. The major precursor observed, p45, acquires the O-linked oligosaccharides relatively slowly to give the mature G protein (Fennie et al. 1985). The failure to identify the precursor to G in PVM prevented the demonstration of a similar or different mechanism of processing for the PVM G protein.

The possibility of a glycoprotein equivalent to the F polypeptide of RS virus occurring in PVM was considered. The evidence presented for such a polypeptide suggested that it was poorly glycosylated being more readily labelled with [^{35}S]-methionine than with [^3H]-glucosamine. The difference in mobility of this polypeptide under reducing and non-reducing conditions suggested that if it exists as two

disulphide bonded fragments then the smaller fragment would only have a M_r of 5,000. No polypeptide with a M_r of this size was detected possibly due to poor labelling or aberrant mobility. The 12K polypeptide was expressed on the cell surface but although it appeared after a chase period in pulse chase experiments this did not correlate with a conversion of a F0 to a F1 polypeptide (the only possible band that could have represented F0 was a 50K polypeptide observed during a pulse chase experiment carried out with ^3H -glucosamine as the label). The F1 polypeptide was observed to be present in a particulate fraction of PVM infected cell supernatants along with the G1 and G2 polypeptides.

A PVM glycoprotein producing a very diffuse band was observed which was similar to a protein of similar mobility in TRT virus and to a glycosylated form of the RS virus protein NS1A.

3.4 Non-glycosylated PVM polypeptides

3.4.1 Introduction

Most of the work described has concerned studies on the glycoproteins of PVM. This section describes some studies carried out on the non-glycosylated PVM polypeptides. These experiments fell into three main categories: experiments on the N and 39K polypeptides of PVM, comparison of the relationships of the other non-glycosylated PVM polypeptides by partial proteolysis, and protein phosphorylation in viruses of the genus *Pneumovirus*.

Studies on the nucleocapsid polypeptide of PVM addressed two questions. Firstly, experiments described in the preceding section described the presence of a polypeptide with the mobility of the PVM nucleocapsid or 39K polypeptide on the surface of infected cells. Experiments described in this section showed that the detection of this polypeptide was an artefact due to the conditions used for the immunoprecipitation. Secondly, Cash et al. (1979b) observed a 38K polypeptide of RS virus migrating slightly faster than the nucleocapsid polypeptide that was related to it, so it was of interest to find out if the 39K polypeptide of PVM showed a similar relationship to the nucleocapsid polypeptide.

The PVM polypeptides were analysed by partial proteolysis with chymotrypsin to look for any possible relationships between them. The relatively large number of PVM associated polypeptides identified in Section 3.1

compared with the number of polypeptides associated with RS virus suggested that some of the PVM polypeptides may have been related. The results of the partial digests suggested that 39K and 35K and the 20K and 19K proteins are related. The M polypeptide and the two M2 like polypeptides of RS virus were also subjected to partial proteolysis to obtain evidence that the two M2 polypeptides were in fact related.

RS virus has been reported to possess one major phosphorylated polypeptide. This however has only been reported for one subgroup B strain. The phosphorylated polypeptides of PVM have not been described. Immunoprecipitation of 32 phosphorous labelled polypeptides of the RSS-2 strain of RS virus (a subgroup A strain) and of PVM was carried out to identify the phosphorylated polypeptides of these viruses.

3.4.2 Immunoprecipitation of polypeptides expressed on the cell surface using monoclonal antibodies

Monoclonal antibodies were used to immunoprecipitate radiolabelled polypeptides from the surface of virus infected cells. The results are shown in Figure 56. All of the antibodies immunoprecipitated the N/39K polypeptide from total cell lysates. In the case of monoclonal antibody 26/4/C4 this was due to the antibody recognizing the 39K polypeptide and the L and 35K polypeptides were co-precipitated as previously described (Section 3.2.4). Antibody 19/1/C9 immunoprecipitated G1 (G2 was not apparent on this gel probably due to poor labelling) and the N/39K

Figure 56 Polypeptides immunoprecipitated by monoclonal antibodies following binding to intact cells

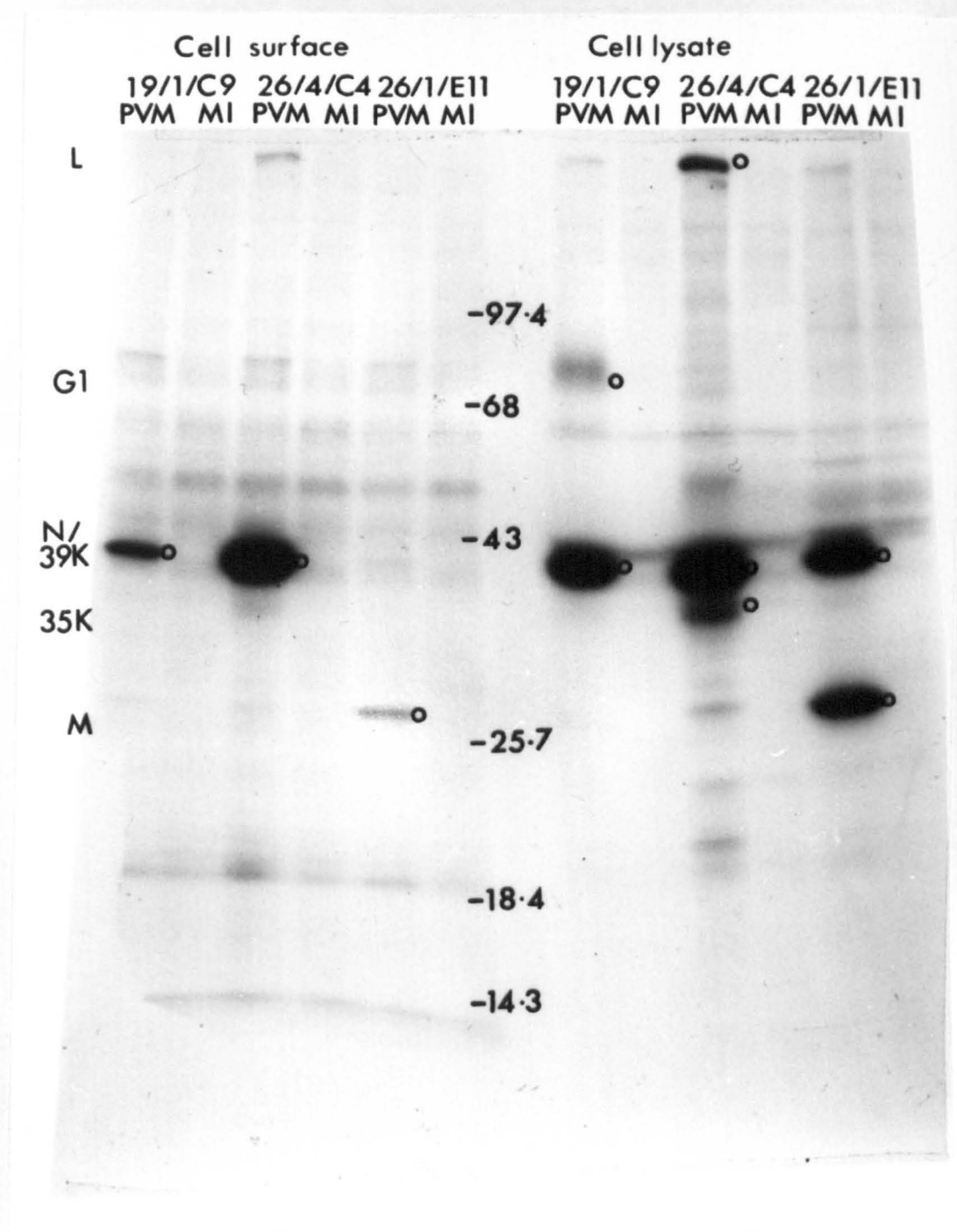
Monoclonal antibodies 19/1/C9, 26/4/C4 and 26/1/E11 directed against the G, 38K and M proteins respectively of PVM were used to immunoprecipitate polypeptides from intact or lysed PVM or mock infected cells. The cells were labelled with [^{35}S]-methionine (100 μCi per ml). The figure shows the immunoprecipitates analysed by SDS-PAGE using a 6 to 15 per cent gradient gel followed by fluorography. The antibodies used are indicated above each pair of tracks and PVM specific polypeptides labelled on the left. The positions of marker polypeptides ($M_r \times 10^{-3}$) is indicated in the centre track.

Cell surface- Polypeptides expressed on the cell surface were immunoprecipitated as described in the legend to Figure 39.

Cell lysates- Cell lysates were immunoprecipitated as described in Section 2.5.15.

PVM- Immunoprecipitates of PVM infected cells.

MI- Immunoprecipitates of mock infected cells.



polypeptide. Antibody 26/1/E11 immunoprecipitated the M polypeptide and the N/39K polypeptide. The M polypeptide appeared not to be expressed on the cell surface since much less label was associated with this polypeptide precipitated from the surface of the cells than from total cell lysates. Monoclonal antibody 19/1/C9 failed to immunoprecipitate G1 or G2 from the cell surface probably due to poor labelling of these polypeptides. Antibody 19/1/C9 did immunoprecipitate the N/39K polypeptide probably due to co-precipitation with an undetectable amount of G1. The 39K polypeptide was immunoprecipitated from the surface of infected cells by monoclonal antibody 26/4/C4. However the results described in the following two sections revealed that this was an artefactual result, non-specific precipitation of the N/39K polypeptide occurring when a buffer with a high salt concentration was used. The results obtained using this approach must therefore be interpreted with care.

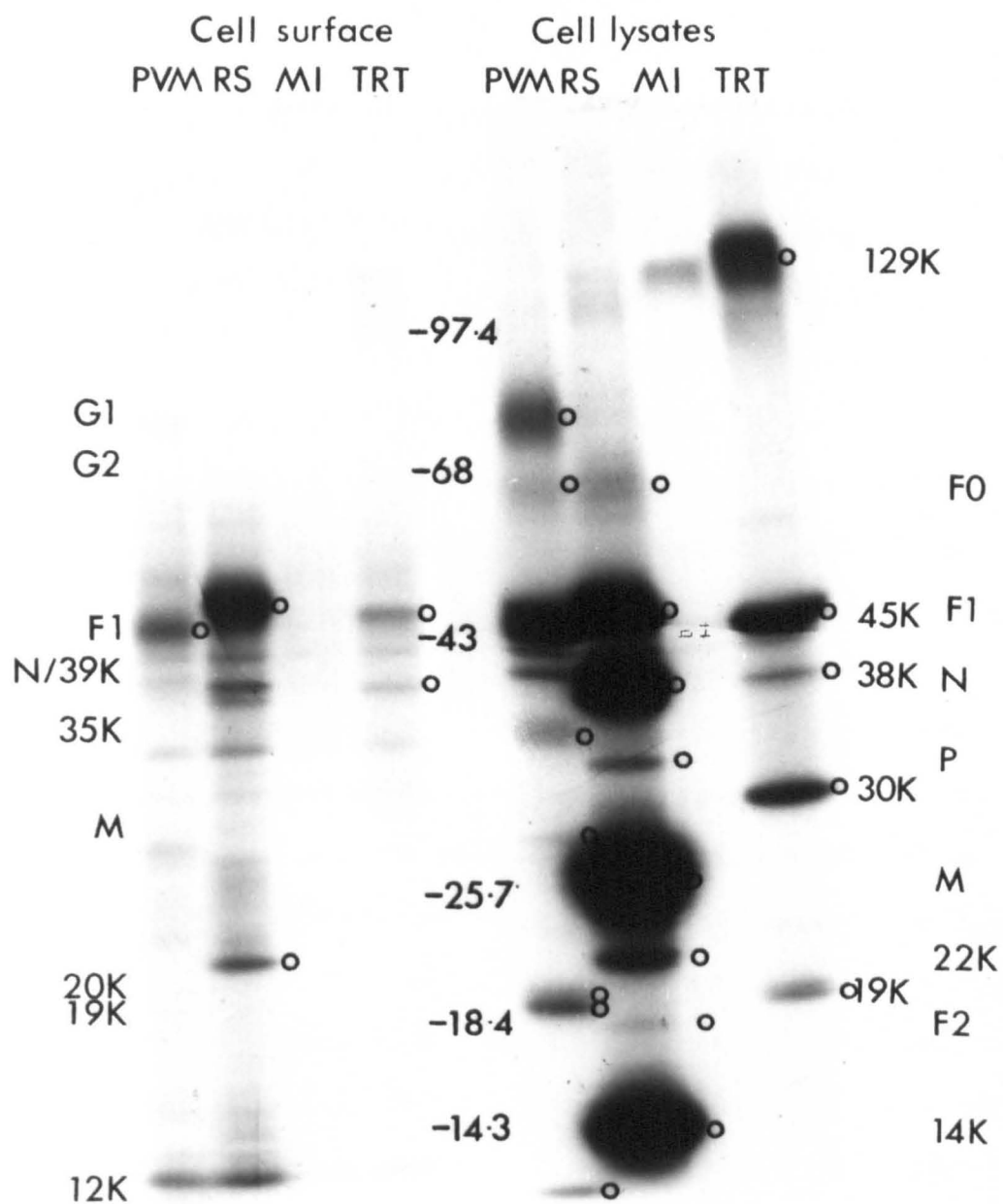
3.4.3 Absence of evidence for expression of the PVM 39K polypeptide on the cell surface by FACS analysis

In order to study the expression of the polypeptide recognized by monoclonal antibody 26/4/C4 on the cell surface by an alternative procedure to that described above, FACS analysis was used. This procedure was used to determine the mean fluorescence intensity of cells following incubation with monoclonal antibodies followed by fluorescein conjugated anti-mouse immunoglobulins. Three

**Table 16 FACS analysis of PVM polypeptides expressed on the
surface of infected cells**

Antibody	Dilution	Mean fluorescence with	
		PVM infected cells	mock infected cells
anti-PVM	1/10	214.14	33.26
	1/50	81.46	12.48
	1/250	24.14	4.24
19/1/C9	1/10	157.14	10.13
(anti-G1/G2)	1/50	145.36	9.46
	1/250	126.56	2.42
26/4/C4	1/10	10.07	7.89
(anti-39K)	1/50	8.10	5.15
	1/250	4.35	2.74
26/1/A2	1/10	9.20	14.83
(anti-N/39K)	1/50	5.87	9.02
	1/250	3.96	8.19
26/1/E11	1/10	14.70	13.46
(anti-M)	1/50	5.43	6.08
	1/250	3.06	2.93

different antibody concentrations were used to determine if the antibody level was saturating. The results are shown in Table 16. Positive results are indicated in bold type. The anti-PVM serum and monoclonal antibody 19/1/C9 were used as positive controls and the anti-M monoclonal antibody 26/1/E11 as a negative control. Antibody 19/1/C9 was used as a positive control since although it did not precipitate polypeptides from the cell surface it did give a positive reaction in immunofluorescence experiments (Figure 17). It can be seen that the negative control (using monoclonal antibody 26/1/E11) did not give significant levels of fluorescence. The anti-PVM serum control did not appear to reach saturating levels of antibody, the mean fluorescence increasing with increasing antibody concentration. The 19/1/C9 monoclonal antibody gave similar mean fluorescence levels at each concentration and so may have been saturating although the conjugate concentration could have been limiting. (The slight increase in fluorescence with increasing antibody concentration was less than 1.5 fold for a 25 fold increase in antibody concentration and was not considered significant.) It can be seen that neither antibody 26/4/C4 nor antibody 26/1/A2 gave levels of fluorescence above those for the negative control so the 39K polypeptide did not appear to be expressed on the cell surface.



3.4.4 Immunoprecipitation of cell surface polypeptides using a low salt buffer

A systematic study of the use of buffers with different pH values and salt concentrations was carried out during an attempt to optimise immunoprecipitation with the monoclonal antibody 19/1/C9. This antibody was found not to immunoprecipitate any polypeptides on many occasions. This was subsequently found to correlate with the virus stocks used and was probably due to some stocks giving insufficient multiplicities of infection to enable the G polypeptides to be labelled adequately. The experiment revealed that the background level of label incorporation decreased with increasing salt concentration of the buffer. However a PVM polypeptide (either N or the 39K polypeptide) showed anomalous behaviour and the band intensity increased with increasing salt concentration. This suggested that the use of buffer with a high salt concentration might affect the results of immunoprecipitation experiments. Anomalous precipitation could account for the apparent expression of the N/39K polypeptide on the cell surface when immunoprecipitation of intact cells was used. A cell surface immunoprecipitation using a buffer with a lower salt concentration was therefore carried out. The result is shown in Figure 57, the composition of the buffer being 10mM Tris-HCl (pH7.4), SDS (0.1 per cent w/v), Sodium deoxycholate (1 per cent w/v), Triton X100 (1 per cent v/v) and Sodium chloride (0.15M). It can be seen that the N/39K polypeptide is not precipitated from the surface of intact

cells under these conditions. Similarly only F1 and a 22K polypeptide (which may represent a fragment of F1 like that described with viruses of RS virus by Norrby et al., 1986) were immunoprecipitated from the surface of RS virus infected cells. It was concluded from these data that only the F1 polypeptide of PVM and the F1 related polypeptides of RS virus could be considered to be genuinely expressed on the cell surface in these immunoprecipitation experiments. The figure also shows immunoprecipitates of TRT virus polypeptides that are considered later (Section 3.5.6).

3.4.5 The absence of mobility changes of the 39K polypeptide during pulse chase experiments

Figure 58 shows immunoprecipitates of a pulse chase experiment using the monoclonal antibody 26/4/C4. Insufficient label incorporation was achieved to detect this polypeptide in the supernatant. Two polypeptides of similar mobility can be observed, the lower mobility band probably representing co-precipitating N polypeptide. There is no evidence from this data of any processing as was observed with the N and 38K polypeptides of RS virus (Cash et al., 1979b).

3.4.6 Identification of 39K related polypeptides by analysis of partial chymotryptic digests of PVM polypeptides

All of the PVM polypeptides that could be sufficiently well labelled with [³⁵S]-methionine were digested with chymotrypsin and the products analysed by SDS-PAGE as

**Figure 58 Pulse chase experiment- immunoprecipitation with
anti-39K monoclonal antibody**

PVM infected (PVM) and mock infected (MI) BS-C-1 cells were pulse labelled 48 hours after infection with [^{35}S]-methionine (400 μCi per ml). Pulse labelled samples (P in the figure) were then harvested whilst the other samples were chased in the absence of labelled methionine as described in Section 2.5.13 for the times indicated (in minutes). Cell lysates were immunoprecipitated with monoclonal antibody 26/4/C4. The immunoprecipitates were analysed by SDS-PAGE and fluorography. The fluorograph is shown in the figure. The 39K polypeptide is labelled on the left. The M_r s of the markers ($\times 10^{-3}$) are shown in the figure.

P 10 PVM 20 40 100 MI P 100

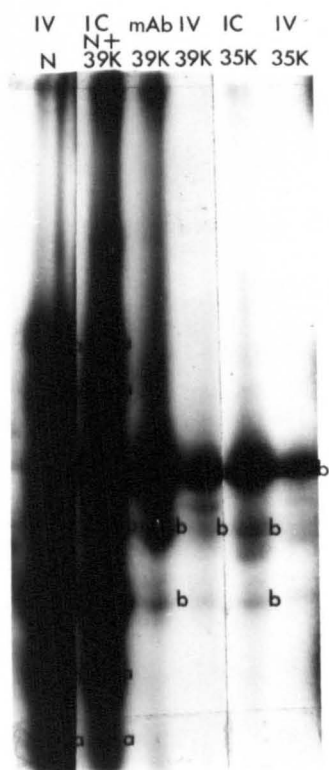
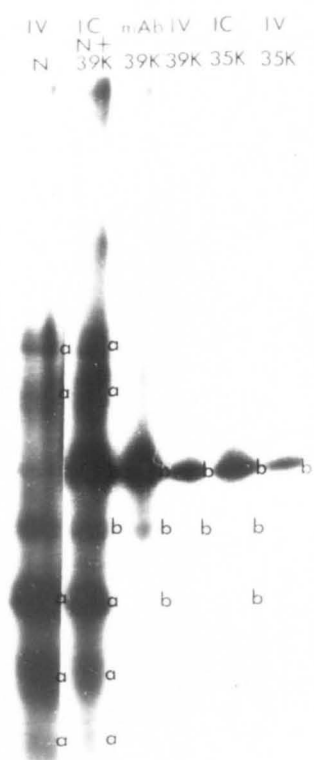
^N
39K



Figure 59 Partial digests of the F1, N, 39K and 35K
polypeptides of PVM

PVM infected cells were labelled from 48 to 72 hours post infection with [^{35}S]-methionine (400 μCi per ml) and immunoprecipitated as described in Section 2.5.15. PVM polypeptides were synthesized *in vitro* as described in Section 2.5.27) and some of the samples immunoprecipitated whilst others were boiled in sample buffer. The samples were analysed by SDS-PAGE and the bands excised. The bands were digested during stacking in a second gel using chymotrypsin (1mg per ml) as described in Section 2.5.22. This gel had a 15 per cent resolving gel. The polypeptide used is indicated above each track. Similar polypeptide profiles are indicated by similar letters to the right of each band. Two prints are shown to enable bands of different intensities to be identified.

- IC- Polypeptide derived from immunoprecipitate of infected cell lysate.
- IV- Polypeptide derived from *in vitro* translation of RNA from PVM infected cells.
- IVI- Polypeptide derived from immunoprecipitate using anti-PVM serum of PVM polypeptides labelled *in vitro*.
- mAb- Polypeptide immunoprecipitated using monoclonal antibody 26/4/C4.



described in the legend of Figure 59. The results are shown in Figures 59 and 60. The N and 39K polypeptides could not be clearly separated when immunoprecipitated from samples labelled *in vivo*. However the polypeptides could be more readily separated when they were labelled *in vitro*. The reason for this difference is not clear. The digests of the N and 39K polypeptides labelled *in vitro* (IV) show different peptides (peptides associated with N are labelled a and those associated with the 39K protein are labelled b in Figure 59). A digest of the N/39K band observed when the polypeptides were labelled *in vivo* (IC) shows the peptides associated with digests of both the N and 39K proteins labelled *in vitro*. The two polypeptides were therefore considered to be distinct. The polypeptide immunoprecipitated by monoclonal antibody 26/4/C4 (mAb in Figure 59) showed a polypeptide pattern similar to that of the 39K polypeptide and the antibody was considered to be specific for the 39K polypeptide on this basis. It was not possible to determine the specificity of this antibody unequivocally on the basis of mobility since the N and 39K polypeptides had such similar mobilities. It was observed that the 35K polypeptide also shared peptides with the 39K polypeptide and may therefore be related to it in some way. The nature of the relationship if it is genuine is not clear. The 25K polypeptide labelled *in vitro* showed a single band that may have corresponded to the major digestion product of the 39K polypeptide (Figure 60) but this was run on a different gel so this was not clear. In

Figure 60 Partial proteolysis of the PVM M, 25K, 24K, 20K and 19K polypeptides.

PVM polypeptides were labelled and separated and digested with chymotrypsin as described in the legend to Figure 59. Two prints are shown to enable bands with different intensities to be identified.

mAb	Polypeptide immunoprecipitated with monoclonal antibody 26/1/E11.
IC-	Polypeptide labelled <i>in vivo</i> and immunoprecipitated with anti-PVM serum.
IV-	Polypeptide labelled <i>in vitro</i> .
IVI-	Polypeptide labelled <i>in vitro</i> and immunoprecipitated with anti-PVM serum.

IC mAb IV IV IV IV IC IC
M M M 25K 24K M 25K 20K 19K

a a a a a
b b c c
a a a a a
a a a a a

IC mAb IV IV IV IV IC IC
M M M 25K 24K M 25K 20K 19K

a a a a a
b b c c
a a a a a
a a a a a

contrast to the situation with RS virus the N and 39K polypeptides are not related. Polypeptides of 35K and 25K may be related to the 39K polypeptide.

3.4.7 Identification of other possible relationships between PVM polypeptides with different mobilities

Figure 60 shows that digestion of the M polypeptide of PVM with chymotrypsin gave a similar peptide pattern regardless of whether the labelling was carried out *in vivo* (IC) or *in vitro* (IV). Digestion of the M polypeptide labelled *in vitro* and immunoprecipitated with monoclonal antibody 26/1/E11 (mAb) gave a similar peptide pattern to that observed with the M polypeptide immunoprecipitated using anti-PVM serum (IVI). Partial digests of the 24K polypeptide and the M polypeptide shared peptides of similar mobilities suggesting that the polypeptides may have been related although the peptide patterns were not identical. The 20K polypeptide and the 19K polypeptide shared a similar pattern of peptides and may therefore not be unique polypeptides. The possibility exists in this case that the bands were not sufficiently well resolved to cut each one out without extensive cross contamination with labelled protein from the other.

3.4.8 Labelling of pneumovirus polypeptides with ³²phosphorous

RS virus has been shown to have one major phosphorylated polypeptide, the P protein (Cash et al.

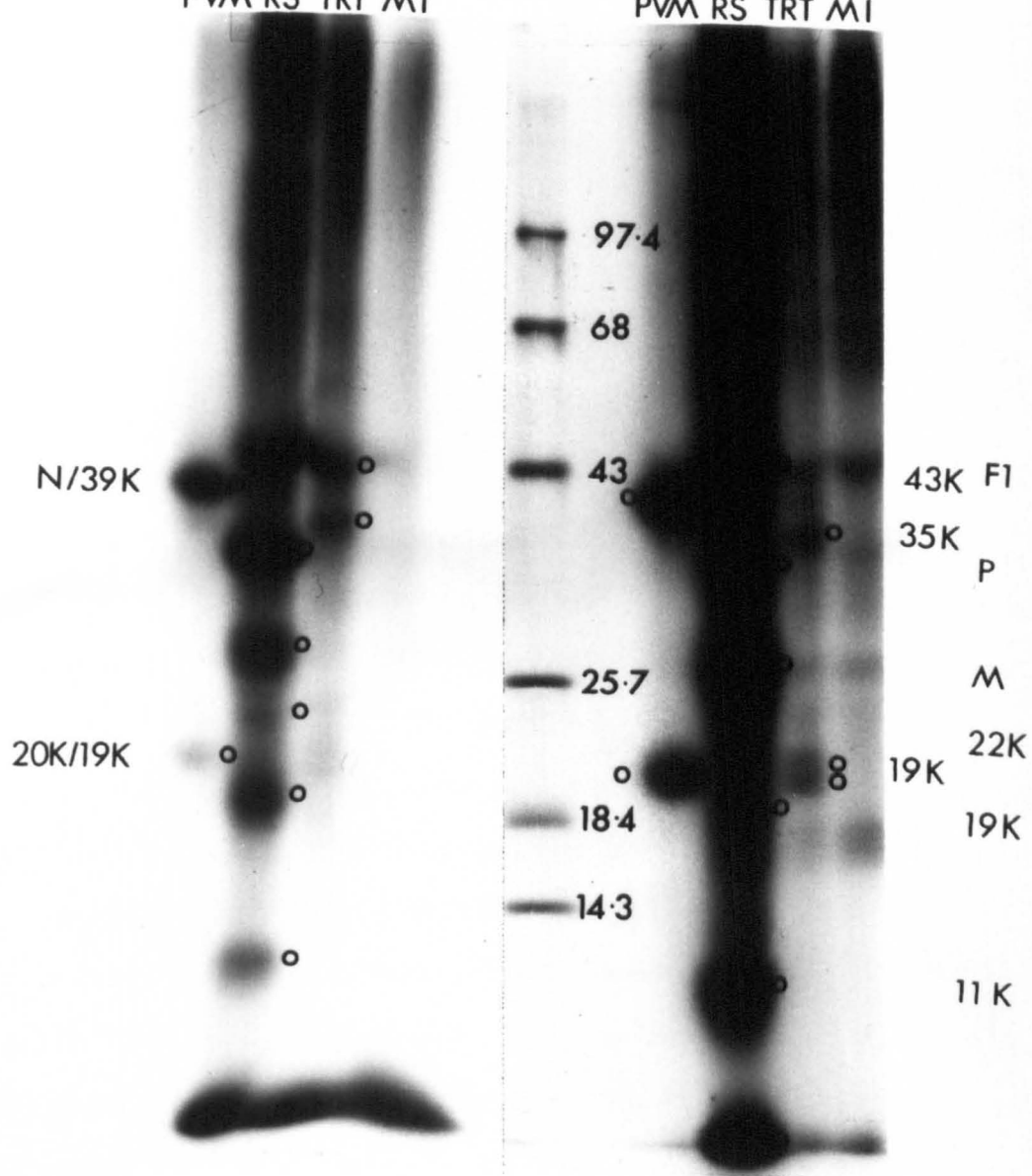
Figure 61 Immunoprecipitation of pneumovirus polypeptides
labelled with 32 phosphorous.

PVM, RS virus, TRT virus and mock infected cells were incubated in phosphate free medium from 48 to 52 hours post infection. The cells were then incubated overnight in similar medium containing 32 phosphorous (500 μ Ci per ml). The cells and supernatants were harvested as described in Section 2.5.12 and immunoprecipitated with the homologous antiviral sera. The samples were analysed by SDS-PAGE followed by autoradiography using an intensifying screen. The autoradiograph is shown in the figure. The sizes of the markers ($M_r \times 10^{-3}$) are indicated in the centre. PVM specific polypeptides are labelled on the left, RS virus polypeptides on the right and TRT virus polypeptides on the far right.

Cell lysate-Samples immunoprecipitated from cell lysates	
PEG-	Polypeptides immunoprecipitated from PEG precipitates of cell supernatants
PVM-	Immunoprecipitates of samples from PVM infected cells.
RS-	Immunoprecipitates of samples from RS virus infected cells.
TRT-	Immunoprecipitates of samples from TRT virus infected cells.
MI-	Immunoprecipitates of samples from mock infected cells carried out using all of the antiviral sera together.

Cell lysate
PVM RS TRT MI

PEG
PVM RS TRT MI



1979b). Phosphoproteins of PVM and TRT virus have not been described. In order to identify any phosphorylated polypeptides of PVM and TRT virus and to confirm the result observed for RS virus using a more sensitive and specific technique immunoprecipitation was used. PVM, RS virus and TRT virus infected cells were labelled with 32 phosphate and the cell lysates and PEG precipitates of the supernatants were then immunoprecipitated with the appropriate antisera. The phosphoproteins of TRT virus are described in Section 3.5.7. Mock infected cells were immunoprecipitated with all the antisera in combination as a control. The polypeptides immunoprecipitated were analysed by SDS-PAGE and the results are shown in Figure 61. Label migrating at the top of the gel may have represented phosphorylation of the L polypeptide of RS virus but it was very diffuse and probably represented non-specifically precipitated material. The label at the bottom of the gel may also have represented non-specifically precipitated material. In the case of RS virus a band at the bottom of the PEG precipitate track had no counterpart in the tracks of PEG precipitates of supernatants of the other cells. Label in the 60K to 150K region of the gel could not be correlated with RS virus polypeptides except possibly for the G polypeptide. A band occurred in the region of the RS virus F1 polypeptide although this band was not clearly virus specific since there was a band in a similar position on the track of immunoprecipitates of mock infected cells. The P protein of RS virus was the most heavily labelled band in

immunoprecipitates of cell lysates. A polypeptide migrating in the position of the M polypeptide of RS virus was phosphorylated. A low intensity band was observed in the region of the 22K RS virus polypeptide which was not detected amongst the polypeptides in the PEG precipitate. A polypeptide of 19K was observed that could have represented F2. A polypeptide of 11K may have represented the 11K non-structural protein of RS virus. However this polypeptide was present in the supernatants of infected cells in this experiment. As described above it was not clear if the band at the bottom of the gel was virus specific. This band would represent a polypeptide with an M_r of only about 7,000. This would be smaller than any of the reported RS virus polypeptides and so the band may represent a fragment of a larger polypeptide or non-specifically precipitated material.

Only two PVM specific bands were observed. One of the phosphoproteins was the N and/or 39K polypeptide whilst the other was the 20K or 19K polypeptide.

The mobilities of PVM and RS virus phosphoproteins are therefore different. The nature of the phosphoproteins of higher mobility than P is not known. Experiments with monoclonal antibodies would be required to determine which of the higher mobility polypeptides is phosphorylated. The 26/4/C4 monoclonal antibody binds to both the PVM 39K polypeptide and the RS virus P protein so these polypeptides could be analogous proteins.

3.4.9 Summary

The results presented in this section describe some of the properties of the non-glycosylated polypeptides of PVM. It was not possible to distinguish the N and 39K polypeptides of PVM in many experiments. The two polypeptides appeared to be distinct giving different patterns of peptides after digestion with chymotrypsin and trypsin. The 39K polypeptide could be distinguished from the N polypeptide when the monoclonal antibody 26/4/C4 was used (Section 3.4.6). The similar mobility of the N and 39K polypeptides has prevented the unambiguous determination of which of these polypeptides is phosphorylated. Results described in the previous section (3.3.15) suggested that either the N or the 39K polypeptide was expressed on the cell surface. This result was shown to be an artefact due to the salt concentration used during the immunoprecipitation. The 39K and 35K polypeptides appeared to be related. The 35K polypeptide did not appear to be phosphorylated so if the 39K polypeptide is the phosphoprotein the 35K polypeptide may represent its unphosphorylated form. There may be differences in the epitope recognized by monoclonal antibody 26/4/C4 between the 39K and 35K polypeptides since the latter polypeptide was only weakly precipitated by the monoclonal antibody and was not detected on Western blots. The antibody may recognize an epitope involving phosphate groups since it also binds to the RS virus phosphoprotein (Section 3.2.5). It is not known if the antibody immunoprecipitates any TRT virus polypeptides. If the 39K

and 35K polypeptides are in fact related the absence of the 35K polypeptide in PVM infected BHK-21 cells (Cash et al . 1979) may be due to the absence of that form of the polypeptide in these cells.

The M protein of PVM did not appear to be phosphorylated unlike the RS virus M protein which had a similar mobility and charge. The 25K and 24K PVM polypeptides synthesized *in vitro* may not represent unique polypeptides. One of the bands shared peptides with the 39K polypeptide whilst the other shared peptides with the M polypeptide following digestion with chymotrypsin.

The second phosphoprotein observed with PVM had no clear counterpart in RS virus. This polypeptide may have been either the 20K or the 19K polypeptide (or both since they were not always well resolved). A 19K phosphoprotein was observed with RS virus but the only protein in this area of the gel was F2 and there is no evidence that the 20K and 19K were F2-like proteins. The 20K/19K phosphoprotein of PVM was not therefore considered to be analogous to the 19K polypeptide of RS virus. The 20K and 19K polypeptides appeared to show similar peptide patterns after digestion with chymotrypsin but the difficulty in separating these polypeptides may have resulted in their being heavily cross contaminated. The 18K, 16K and 12K polypeptides of PVM could not be compared with the other PVM polypeptides satisfactorily by partial proteolysis due to their high mobilities and poor labelling.

3.5 Polypeptides of TRT virus

3.5.1 Introduction

The polypeptides of TRT virus have not been characterized in any detail. The polypeptides observed on stained gels of partially purified virus have been described (Collins & Gough 1988). To obtain additional information on the viral polypeptides radiolabelling was used to identify the polypeptides synthesized in infected cells. This approach was hindered by the high background of cellular protein synthesis even in the presence of actinomycin D. The study was therefore extended using *in vitro* translation of RNA from TRT virus infected cells. A murine antiserum was also produced to enable immunoprecipitation of virus specific polypeptides to be carried out. The use of [32 P]phosphate and [3 H]-glucosamine labelling combined with immunoprecipitation enabled the phosphorylated and glycosylated viral polypeptides to be identified.

3.5.2. TRT virus induced polypeptides observed in infected cells

Figure 62 shows that five virus specific polypeptides with calculated M_r s of 38K, 35K, 30K, 19K and 15K could be identified in [35 S]-methionine labelled TRT virus infected cell lysates. The cells were infected as described in the legend to Figure 62. These three polypeptides were probably equivalent to the 49K, 45K and 39K polypeptides described by Collins et al. (1986b). The relative molecular mass estimates given by these authors were subsequently revised

Figure 62 Polypeptides of TRT virus synthesized in infected cells.

Confluent monolayers of BS-C-1 cells were infected at a multiplicity of infection of 2 and incubated at 33°C for 48 hours before labelling for 24 hours with [³⁵S]-methionine in the presence of actinomycin D (2.5µg per ml). A fluorograph of the TRT virus polypeptides synthesized in infected BS-C-1 cells analysed on a 6-15 per cent gradient resolving gel is shown.

TRTV Samples from TRT virus infected cultures

MI Samples from mock infected cell cultures

CELL
LYSATE
MI TRTV

97.4K-

68K-

43K-

o38K
o35K

o30K

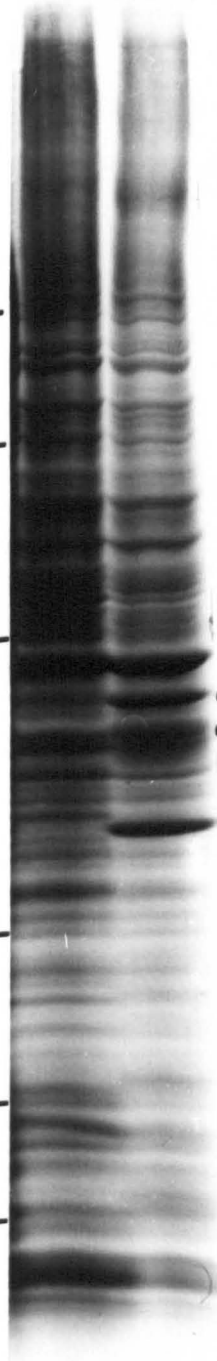
25.7K-

o19K

18.4K-

o15K

14.3K-



downwards (Collins & Gough 1988). The pattern of mobilities was similar but their relative molecular mass estimates differed due to the use of different molecular weight standards.

3.5.3. Analysis of TRT virus polypeptides using two dimensional polyacrylamide gel electrophoresis

[³⁵S]-methionine labelled lysates of TRT virus infected BS-C-1 cells were analysed by two dimensional gel electrophoresis as described in the legend to Figure 63. The two dimensional gels of RS virus (a), PVM (b), and mock infected (c) cell lysates are included in the same figure as that of TRT virus infected cell lysates (d) for comparison. The positions of the virus specific polypeptides are shown superimposed on the autoradiograph of the polypeptides from mock infected cells on the right of each autoradiograph. Acidic polypeptides of 38K, 35K and 15K and a basic polypeptide of 30K were identified in the TRT virus infected cell lysates. These had similar mobilities and charge properties to the N, P, M and 15K polypeptides of RS virus. Like the nucleocapsid proteins of PVM and RS virus some streaking of the TRT virus 38K polypeptide was observed.

3.5.4 TRT virus polypeptides synthesized *in vitro*

TRT virus polypeptides were synthesized *in vitro* using a rabbit reticulocyte lysate system with RNA extracted from TRT virus infected cells incubated in the presence of actinomycin D as described in Sections 2.5.26 and 2.6.27.

Figure 63 Analysis of pneumovirus polypeptides in infected cell lysates on two dimensional gels.

BS-C-1 cells were infected and labelled as described in the legend to Figure 62. The cells were lysed and analysed by non-equilibrium pH gradient electrophoresis followed by SDS-PAGE as described in Section 2.5.17. NEPHGE was carried out in the horizontal direction with the acidic end to the right. Diagrams of the virus specific polypeptides overlaid on the mock infected cell polypeptides are shown adjacent to each autoradiograph.

- a) Autoradiograph of gel of RS virus infected cell lysate.
- b) Autoradiograph of gel of PVM infected cell lysate.
- c) Autoradiograph of gel of mock infected cell lysate.
- d) Autoradiograph of gel of TRT virus infected cell lysate.

BASIC

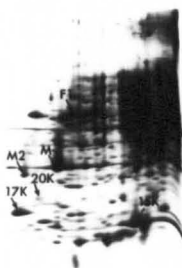
← NEPHGE

ACIDIC

SDS-PAGE

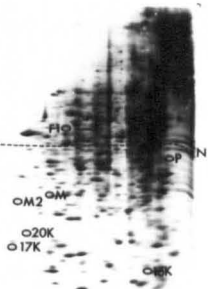
a

-97.4
-68
-43
-25.7
-18.4
-14.3



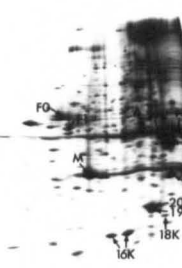
b

-97.4
-68
-43
-25.7
-18.4
-14.3



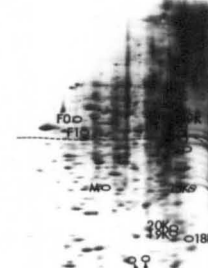
b

-97.4
-68
-43
-25.7
-18.4
-14.3



c

-97.4
-68
-43
-25.7
-18.4
-14.3



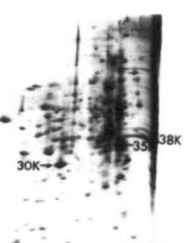
c

-97.4
-68
-43
-25.7
-18.4
-14.3



d

-97.4
-68
-43
-25.7
-18.4
-14.3



e

-97.4
-68
-43
-25.7
-18.4
-14.3



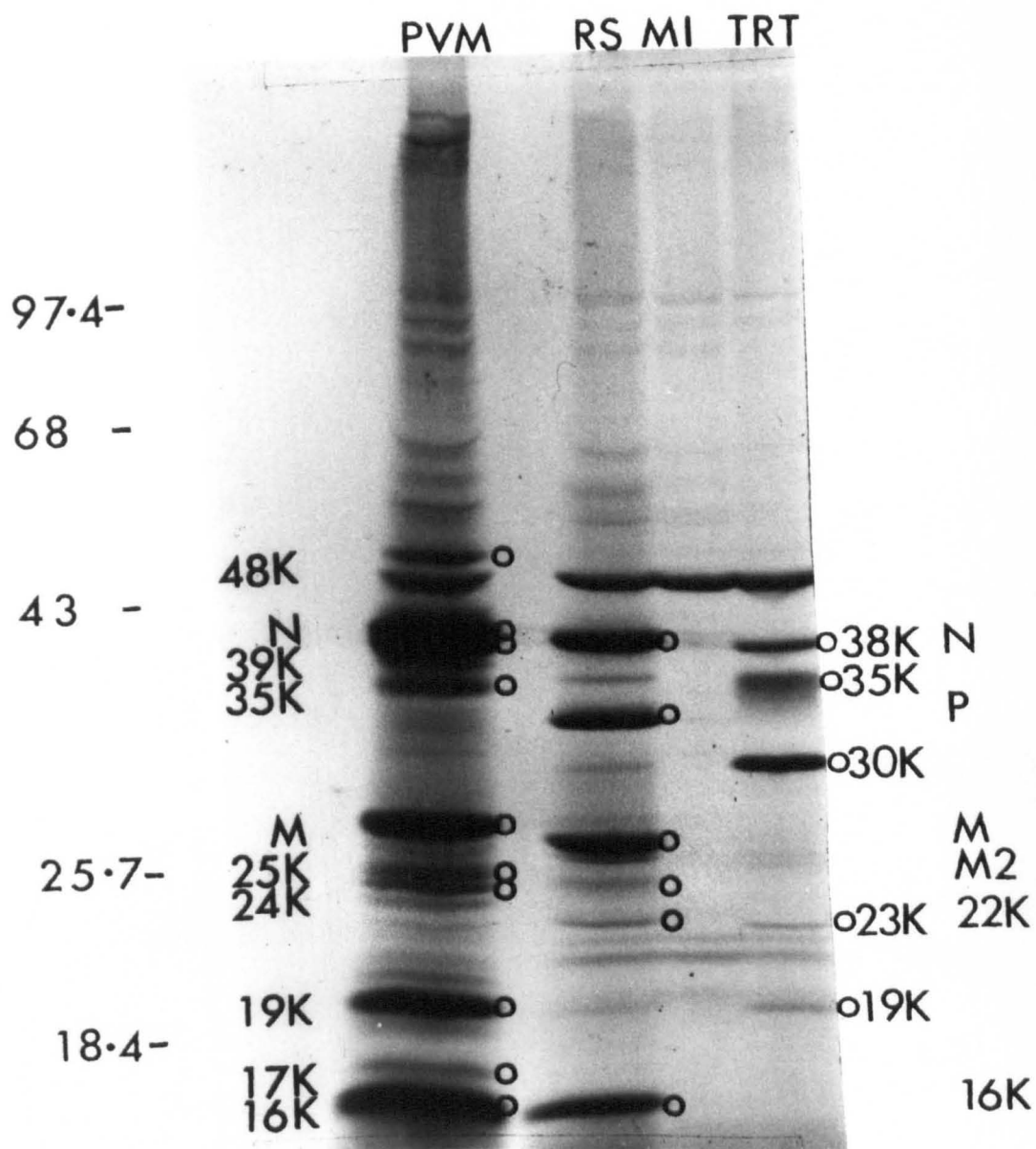
The [^{35}S]-methionine labelled polypeptides separated by SDS-PAGE are shown in Figure 64 along with PVM and RS virus polypeptides translated *in vitro*. The significance of the faint unmarked bands in the PVM and RS virus tracks is not known. It can be seen that the polypeptide profile of TRT virus differs from that of PVM and RS virus to a larger extent than the polypeptide profiles of these two viruses differ from each other. Four of the five TRT virus associated polypeptides identified *in vivo* the 38K, 35K, 30K and 19K polypeptides could be identified *in vitro*. A polypeptide not observed amongst the polypeptides labelled *in vivo* (the 23K polypeptide) was observed amongst their *in vitro* translation products of RNA from TRT virus infected cells. This may have been due to the 23K polypeptide being of low abundance in infected cells or due to it being processed in some way. The 15K polypeptide observed amongst TRT virus specific polypeptides labelled *in vivo* was not observed amongst the polypeptides synthesized *in vitro* on single dimension gels suggesting that it may not have been a primary translation product.

The products of *in vitro* translation of RNA from TRT virus infected cells were also analysed by two dimensional NEPHGE/SDS-PAGE. The results are shown in Figure 65 along with the two dimensional gels of PVM and RS virus polypeptides synthesized *in vitro* using RNA extracted from RS virus (a), PVM (b) and mock infected cells. As had been observed for a polypeptide of similar mobility observed in infected cells (Section 3.5.3.) the 38K polypeptide was

Figure 64 Polypeptides of TRT virus synthesized *in vitro*

RNA was extracted from PVM, RS virus, TRT virus and mock infected cells as described in Section 2.5.26. The RNA was used to direct the synthesis of virus specific polypeptides *in vitro* as described in Section 2.5.27. The figure shows a fluorograph of the polypeptides analysed by SDS-PAGE. The PVM polypeptides are labelled on the left, the TRT virus polypeptides are labelled on the right and the RS virus polypeptides are labelled on the far right. The positions of the polypeptides are indicated by circles to the right of each track. Molecular weight markers ($M_r \times 10^{-3}$) are indicated on the left.

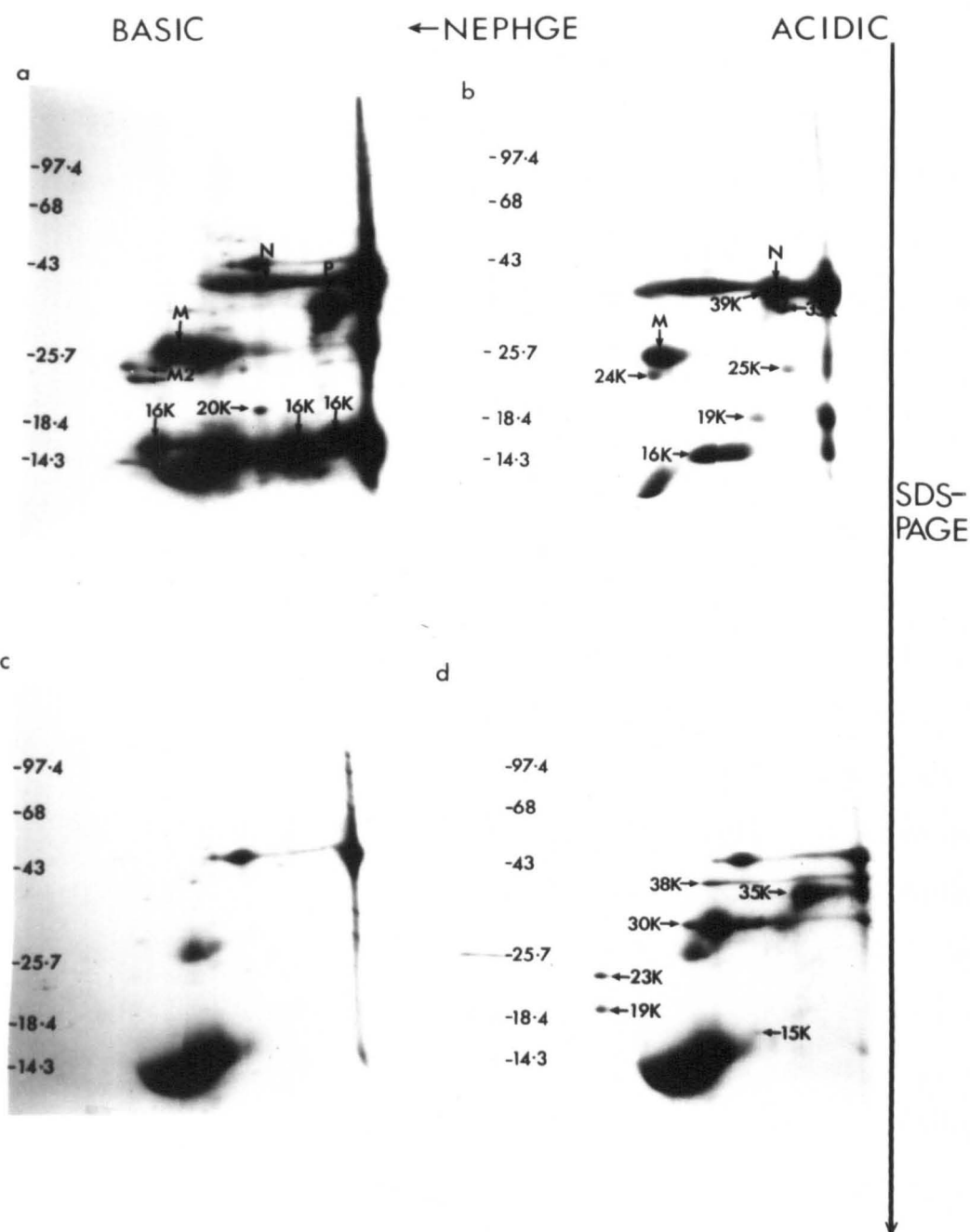
- PVM- Polypeptides synthesized *in vitro* using RNA from PVM infected cells.
- RS- Polypeptides synthesized *in vitro* using RNA from RS virus infected cells.
- MI- Polypeptides synthesized *in vitro* using RNA from uninfected cells.
- TRT- Polypeptides synthesized *in vitro* using RNA from TRT virus infected cells.



**Figure 85 Analysis of pneumovirus polypeptides synthesized
in vitro on two dimensional gels.**

Polypeptides synthesized *in vitro* as described in Section 2.5.26 were analysed by NEPHGE/SDS-PAGE as described in Section 2.5.27. NEPHGE was carried out in the horizontal direction with the acidic end to the right.

- a) Fluorograph of PVM polypeptides synthesized *in vitro* analysed by NEPHGE/SDS-PAGE
- b) Fluorograph of RS polypeptides synthesized *in vitro* analysed by NEPHGE/SDS-PAGE
- c) Fluorograph of mock infected cell polypeptides synthesized *in vitro* analysed by NEPHGE/SDS-PAGE
- d) Fluorograph of TRT virus infected cell polypeptides synthesized *in vitro* analysed by NEPHGE/SDS-PAGE



observed to streak in the first dimension. The 35K and 30K polypeptides were acidic and basic respectively as has been described for polypeptides of similar mobility in infected cells (Section 3.5.3). The 30K polypeptide, like the M polypeptide of RS virus was observed to have a similar mobility to a series of more acidic polypeptides. The 23K and 19K polypeptides were observed to be highly basic. The 15K polypeptide observed amongst *in vitro* translation products of RNA from TRT virus infected cells was more basic than the 15K polypeptide observed in infected cells (Figure 63). This suggests that either these two polypeptides are distinct or that some form of processing occurs *in vivo* altering the charge on this polypeptide.

3.5.5. Cross neutralization tests with antiviral sera

Murine antisera were raised against two types of TRT virus giving different plaque morphologies and used in cross neutralization tests with both types of TRT virus and PVM and RS virus. The two variants of TRT virus produced focal plaques (TRTf) and syncytial plaques (TRTs) as described by Ling & Pringle (1988). Anti-PVM and anti-RS virus sera were also used. The results are shown in Table 17. It can be seen that the murine anti-PVM and bovine anti-RS virus sera showed high neutralizing activity with the homologous virus and no neutralizing activity with heterologous viruses. The murine anti-TRT virus sera showed relatively low neutralizing titres. The anti-TRTf virus serum had higher titres against both forms than the anti-TRTs virus serum.

Table 17 Cross neutralization tests with anti-pneumovirus
sera

Antiserum	Neutralization titre with:-			
	PVM	RS virus	TRT(F) virus	TRT(S) virus
anti-PVM	27,000	<20	<20	<20
anti-RS virus	<20	25,000	<20	<20
anti-TRT(F) virus	120	<20	940	390
anti TRT(S) virus	180	<20	560	240
Pre-immune 1 ¹	90	ND ²	<20	<20
Pre-immune 2 ¹	90	ND ²	<20	<20

1 Pre-immune 1 serum was taken from mice subsequently immunized with TRT(F) virus and pre-immune 2 serum was taken from mice subsequently immunized with TRT(S) virus

2 not done

Both anti-TRT virus sera neutralized the focus forming variant more effectively than the syncytium forming variant. These results suggested that the two viruses were antigenically similar. The sera did not neutralize RS virus but did show very low neutralizing activity with PVM. This low level of anti-PVM activity was also present in pre-immune sera and the level in the anti-TRT virus sera did not correlate with the titres of the two sera against TRT virus. This indicated that there was no cross reactivity between antisera to TRT virus and PVM.

The results show that the two types of TRT virus were related in neutralization tests. PVM, RS virus and TRT virus do not show any cross neutralization reactions.

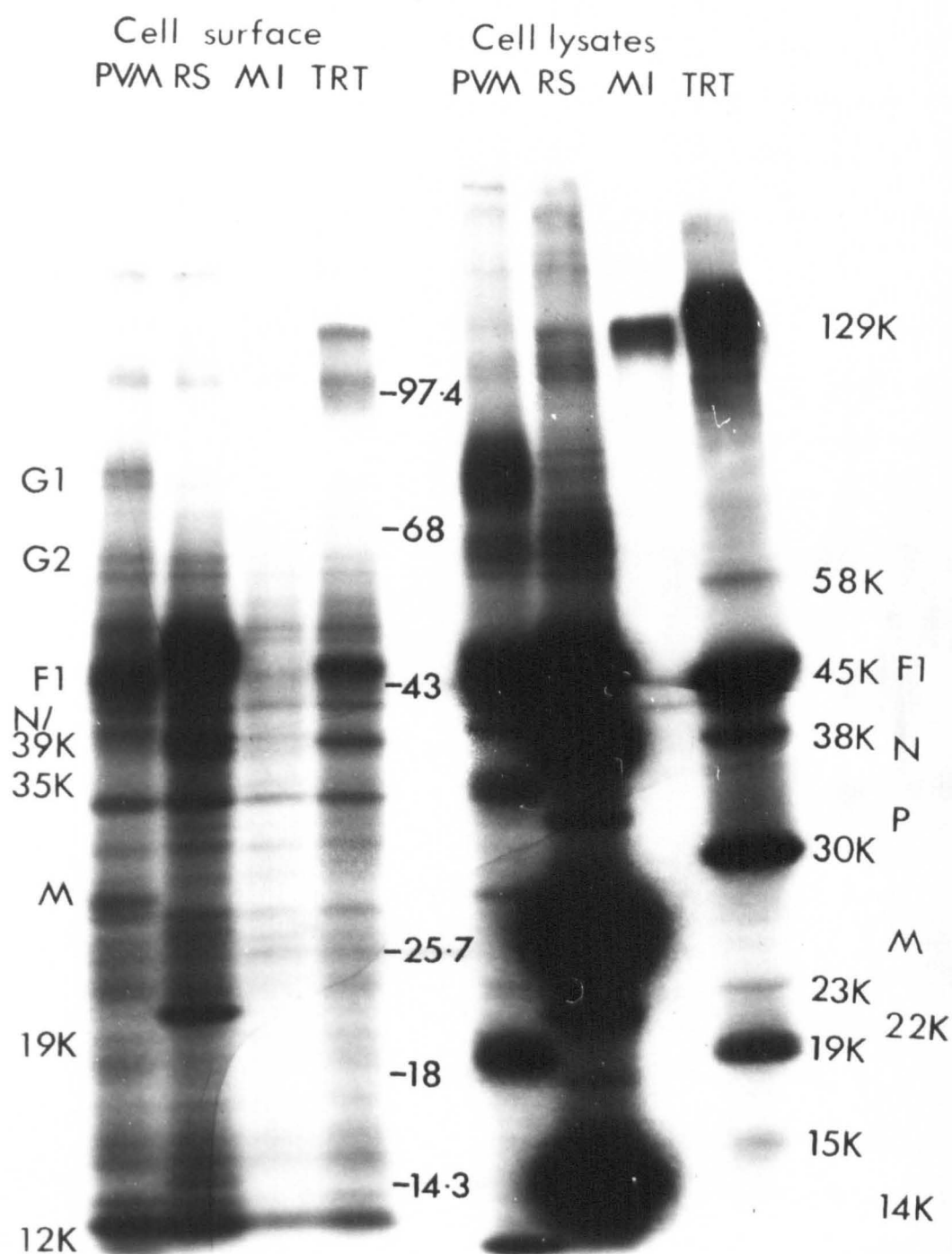
3.5.6 Immunoprecipitation of TRT virus polypeptides with murine anti-TRT virus serum

The anti-TRTf serum described in the preceding section was used to immunoprecipitate TRT virus polypeptides from [³⁵S]-methionine labelled TRT virus infected cell lysates (Figure 66, which is a longer exposure of Figure 57). Eight major polypeptides were immunoprecipitated by the anti-TRTf serum the 129K, 58K, 45K, 38K, 30K, 23K, 19K and 15K polypeptides. A band of similar mobility to the 129K polypeptide was immunoprecipitated from labelled uninfected cells so this polypeptide may not have been virus specific. There also appears to be a 100K polypeptide below the 129K polypeptide. The 19K polypeptide appeared to consist of two closely migrating bands on this gel (only apparent in the

**Figure 66 Immunoprecipitation of TRT virus polypeptides with
a murine antiserum**

Polypeptides were labelled with [^{35}S]-methionine and immunoprecipitated from intact or lysed cells as described in the legend to Figure 57. The polypeptides were immunoprecipitated with the corresponding antiviral sera, the samples from mock infected cells being immunoprecipitated with antisera to all of the viruses. The figure shows a fluorograph of the polypeptides analysed by SDS-PAGE using a 6-15 per cent gradient gel. The PVM specific polypeptides are labelled on the left, the TRT virus polypeptides on the right and the RS virus specific polypeptides on the far right. The positions of the markers ($M_r \times 10^{-3}$) are indicated in the centre.

Cell surface-	Polypeptides immunoprecipitated after binding antibody to intact cells.
Cell lysates-	Polypeptides immunoprecipitated from cell lysates.
PVM-	Polypeptides immunoprecipitated from PVM infected cells.
RS-	Polypeptides immunoprecipitated from RS virus infected cells.
TRT-	Polypeptides immunoprecipitated from TRT virus infected cells.
MI-	Polypeptides immunoprecipitated from mock infected cells.



shorter exposure Figure 57) similar to the 20K and 19K polypeptides of PVM. Figure 57 shows that immunoprecipitation of polypeptides expressed on the cell surface did not clearly reveal any TRT virus specific polypeptides although the 45K and 38K polypeptides may have been present on the cell surface. The TRT virus 35K polypeptide detected in infected cell lysates (e.g. Section 3.5.2) was not precipitated by this antiserum.

If the 129K polypeptide is virus specific it has no counterpart of similar mobility in RS virus or PVM. No G like polypeptide was ever observed in [³⁵S]-methionine labelled cell lysates. The 45K polypeptide has a similar mobility to the F1 polypeptides of PVM and RS virus. Evidence is presented in Sections 3.5.8 and 3.5.9 that this polypeptide and a 58K polypeptide that may represent an F0-like protein are glycosylated. The 38K polypeptide of TRT virus has a similar mobility to the N polypeptides of PVM and RS virus and like them may be associated with nucleocapsids (data not shown).

3.5.7 Phosphorylated polypeptides of TRT virus

³²Phosphate labelled TRT virus polypeptides were immunoprecipitated from infected cell lysates and PEG precipitates of the culture supernatants using the murine anti-TRTf virus serum. The polypeptides were analysed by SDS-PAGE along with the PVM and RS virus phosphoproteins immunoprecipitated with homologous antisera. The results are shown in Figure 61. Three possible phosphoproteins were

immunoprecipitated from samples from TRT virus infected cells. A polypeptide of about 43K was observed to be a stronger band in samples from TRT virus infected cells than a corresponding band in mock infected cells. This polypeptide did not appear to be specifically precipitated from PEG precipitate samples. The other TRT virus phosphoproteins were the 35K and 19K polypeptides. Two polypeptides were identified in the region of the 19K polypeptide as described above (Section 3.5.6). These polypeptides could only be clearly observed in the PEG precipitate sample.

The results of the phosphate labelling studies did not reveal a common pattern of phosphorylation of pneumovirus polypeptides. PVM and RS virus showed a totally distinct pattern of phosphoproteins whilst the pattern with TRT virus contained bands of similar mobility to those of each of the other viruses. The N and 39K polypeptides of PVM were difficult to distinguish due to their similar mobilities. It is likely that the 39K polypeptide is similar to the RS virus P protein because they both bind to monoclonal antibody 26/4/C4. TRT virus has a phosphorylated polypeptide of about 35K that could be analogous to the phosphorylated P protein of RS virus that has a similar mobility. TRT virus also had a possible phosphorylated polypeptide of 43K as did RS virus although a co-migrating polypeptide in uninfected cells precluded a definite identification of these polypeptides as being virus specific. Analysis of ³²P-labelled samples under non-reducing conditions

might establish whether or not the F1 protein of RS virus and the 45K protein of TRT were in fact phosphorylated because the label would then be expected to occur in the F0 and 58K polypeptides. Both PVM and TRT virus had phosphoproteins in the 19K region of the gel which could be equivalent polypeptides.

3.5.8 Identification of TRT virus glycoproteins

Figure 67 shows the proteins present in cell lysates and PEG precipitates of supernatants of TRT virus infected cells labelled with [^3H]-glucosamine and immunoprecipitated with murine anti-TRTf virus antiserum. It can be seen that a very high background of cellular glycoproteins made identification of TRT virus specific glycoproteins difficult in immunoprecipitates of cell lysates. This was due to low multiplicities of infection and the low titre of the antiserum. Putative viral glycoproteins of 95K, 31K and 15K were identified. The situation was clearer with the immunoprecipitates of PEG precipitates of the supernatants. Virus specific polypeptides of 45K, 31K and 15K were readily identified whilst an 83K glycoprotein had a greater intensity than a polypeptide of similar mobility in samples from mock infected cells. The glycoproteins of TRT virus therefore show a similar pattern to those of other pneumoviruses.

**Figure 67 Polypeptides of TRT virus labelled with
[³H]-glucosamine**

TRT virus infected and uninfected BS-C-1 cells were labelled with [³H]-glucosamine (50μCi per ml) from 48 hours to 72 hours post infection. TRT virus infected cells were also incubated in the presence of tunicamycin (2.5μg per ml) and labelled in the presence of the antibiotic in a similar way to the untreated samples. Samples of cell lysates and PEG precipitates of the supernatants (PEG) were immunoprecipitated with murine anti-TRT virus serum as described in Section 2.5.15 and analysed by SDS-PAGE followed by fluorography (Sections 2.5.16. and 2.5.23). The samples were run under both reducing and non-reducing conditions. The figure shows the fluorograph obtained. The positions of the markers are indicated by their M_rs (X10⁻³). TRT virus specific polypeptides are indicated by circles and labelled on the left (for samples run under reducing conditions) and on the right (for samples run under non-reducing conditions) using their M_rs.

Cell lysates- Immunoprecipitates of cell lysates

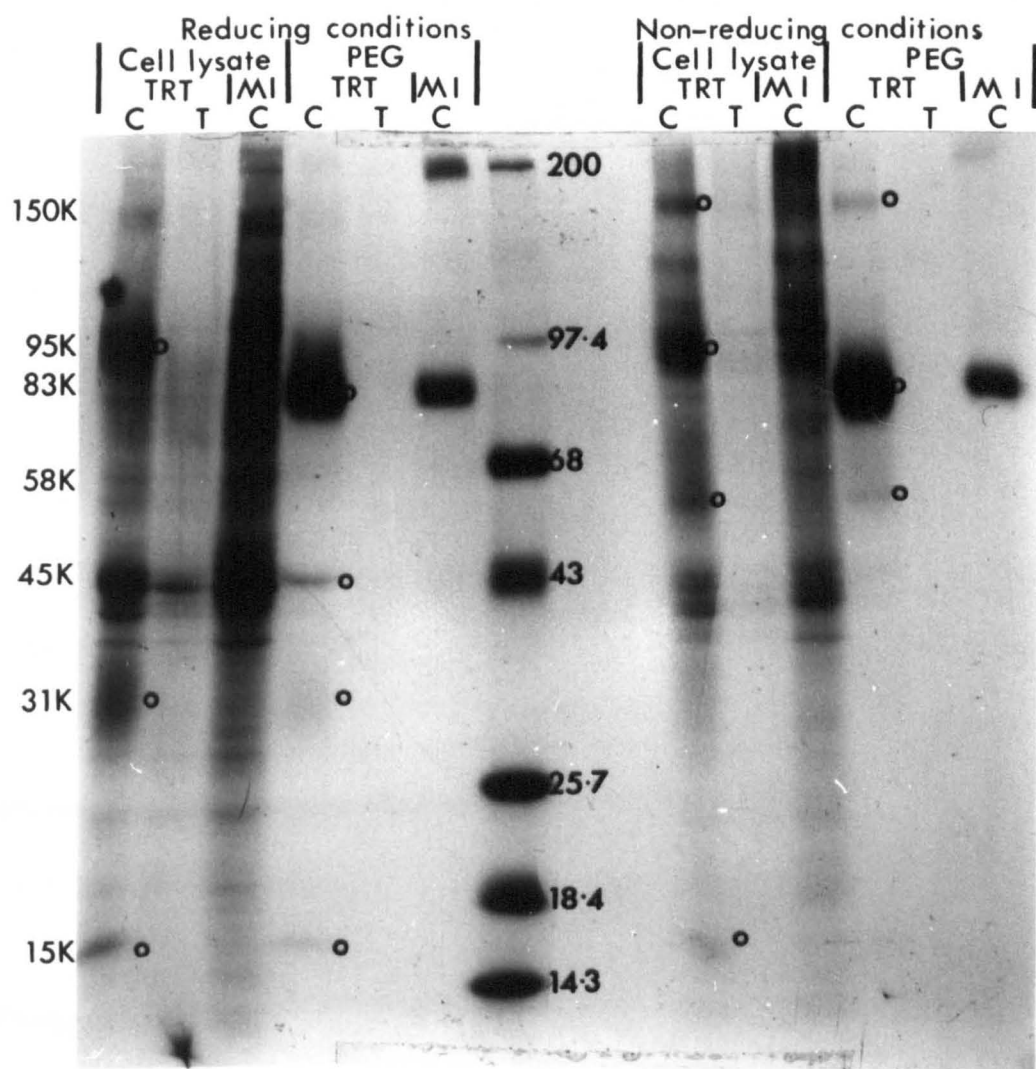
PEG- Immunoprecipitates of PEG precipitates
of cell supernatants

TRT- Samples from TRT virus infected cells

MI- Samples from mock infected cells

T- Samples immunoprecipitated from
tunicamycin treated cells

C- Samples immunoprecipitated from
untreated cells



3.5.9 TRT virus glycoproteins observed under non-reducing conditions

The glycoproteins of TRT virus labelled and immunoprecipitated as described in the preceding section were analysed under non-reducing conditions to identify possible disulphide bonded proteins. The results are shown in Figure 67. Polypeptides of 95K in the cell lysate sample (even less distinct than in samples run under reducing conditions) and 83K in the PEG precipitate showed similar mobilities to those under reducing conditions. The 45K, 31K and 15K polypeptides were all reduced in intensity when the samples were run under non-reducing conditions. Additional virus specific bands of 150K and 58K were observed under non-reducing conditions. The 150K polypeptide may represent aggregates or disulphide bonded multimers of the other viral glycoproteins. The 58K protein may represent an F1,2 like protein, this suggestion being supported by the reduction in intensity of the 45K and 15K polypeptides. Figure 68 shows the results of a similar experiment carried out using [³⁵S]-methionine labelled TRT virus infected cells. It was again observed that the 58K polypeptide band was increased in intensity under non-reducing conditions whilst the 45K polypeptide band was reduced in intensity. The 58K band observed under reducing conditions could, by analogy with the RS virus F protein, have represented the uncleaved F0 like protein of TRT virus.

3.5.10 The effect of tunicamycin on TRT virus glycoprotein synthesis

The effect of tunicamycin treatment of TRT virus infected cells on TRT virus glycoproteins is also shown in Figure 67. It can be seen that no TRT virus specific glycoproteins could be identified in immunoprecipitates of samples from cells incubated in the presence of tunicamycin. This suggests that all of the TRT virus polypeptides have only N-linked oligosaccharides. No unglycosylated [^{35}S]-methionine labelled forms of the 58K or 45K polypeptides could be detected in immunoprecipitates of samples from tunicamycin treated cells (Figure 68). This suggests that they may be unstable in the absence of their N-linked oligosaccharides as was observed for the F protein of subtype B strains of RS virus (Norrby et al. 1986).

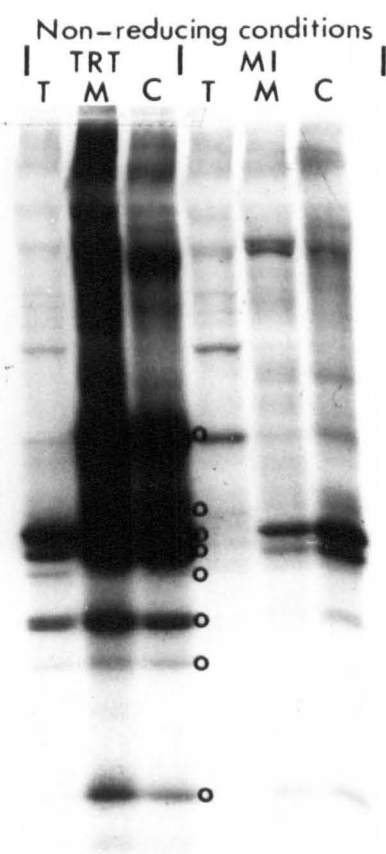
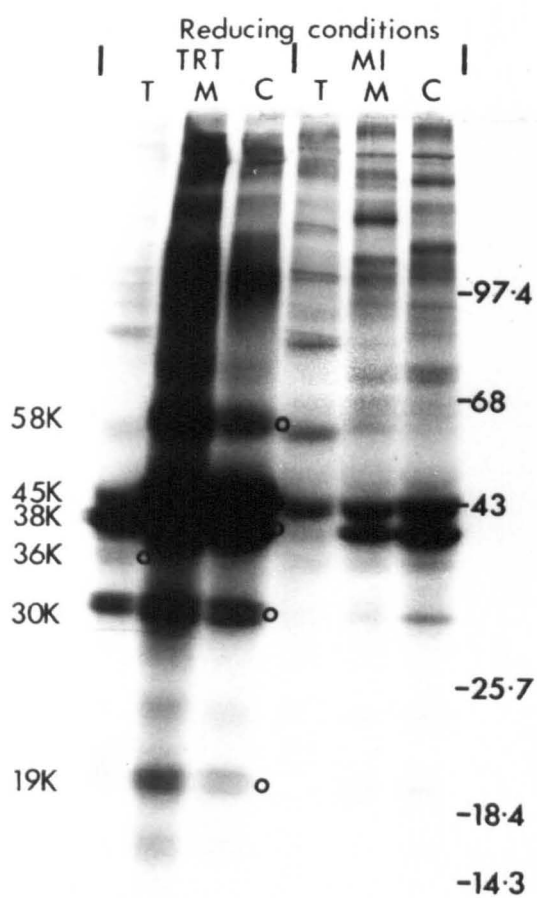
3.5.11 Effect of monensin treatment on synthesis of [^{35}S]-methionine labelled TRT virus specific polypeptides

Immunoprecipitates of [^{35}S]-methionine labelled TRT virus polypeptides immunoprecipitated with murine anti-TRT virus antiserum were analysed by SDS-PAGE and fluorography (Figure 68). The polypeptides were labelled in the presence of tunicamycin (2.5 μg per ml), monensin (0.8 μM) or in the absence of these inhibitors. The bands indicated by circles were considered to be virus specific since they were more intense than bands in the mock infected tracks. The 38K polypeptide unlike co-migrating host cell polypeptides were detected when cells were labelled in the presence of

Figure 68 [^{35}S]-methionine labelled TRT virus polypeptides synthesized in the presence and absence of tunicamycin or monensin

TRT virus infected and mock infected BS-C-1 cells were incubated for 48 hours in the presence or absence of tunicamycin (2.5 μg per ml) or monensin (0.8 μM). The cells were then labelled for 24 hours with [^{35}S]-methionine (50 μCi per ml). Immunoprecipitation with murine anti-TRT virus serum (50 μl per 1×10^6 cells) was used to precipitate TRT virus specific polypeptides that were then resolved by SDS-PAGE under reducing or non-reducing conditions.

- | | |
|------|--|
| TRT- | Immunoprecipitates of TRT virus infected cell lysates. |
| MI - | Immunoprecipitates of mock infected cell lysates. |
| T- | Samples from tunicamycin treated cells. |
| M- | Samples from monensin treated cells. |
| C- | Samples from untreated cells. |



tunicamycin. The apparent resolution of the 38K, 31K and 19K polypeptide bands into two bands each was considered to be an artefact, probably being due to overloading the gel since it was most commonly observed when large amounts of antiserum were used to precipitate viral polypeptides. Two distinct polypeptides were observed in the region of the 38K polypeptide under non-reducing conditions. The relationship between these bands and the polypeptides observed under reducing conditions is not known. The nature of the 36K and 26K polypeptides observed under non-reducing conditions was not clear. The effect of tunicamycin on the viral polypeptides was considered above (Section 3.5.10).

Monensin treatment of TRT virus infected cells only affected the synthesis of the F-like polypeptides. The 58K polypeptide band of TRT virus showed an increased intensity relative to the 45K polypeptide band under reducing conditions. The 58K band was much stronger than the 45K polypeptide band under non-reducing conditions. These results could be explained if a 58K polypeptide was the precursor to a disulphide bonded dimer of a 45K polypeptide and a 15K polypeptide as suggested above (Section 3.5.9). Under non-reducing conditions only a 58K polypeptide would then be observed, whereas under reducing conditions both the 58K and the 45K and 15K polypeptides would be observed if cleavage or reduction of the 58K polypeptide was incomplete. A 15K polypeptide was not observed on fluorographs of the gel shown in Figure 68 even after longer exposures. The relative increase in intensity of the 58K polypeptide band

relative to the 45K polypeptide band when polypeptides were labelled in the presence of monensin could indicate that cleavage of the 58K polypeptide occurred at a site distal to the site of action of monensin as has been observed with the RS virus F protein (Fernie et al. 1985). If this was the case the inhibition of glycoprotein processing by monensin in this experiment was incomplete.

3.5.12 Summary

The results presented in this section describe the identification of the proteins of TRT virus and a partial characterization of their properties. TRT virus like PVM and RS virus probably has a glycoprotein with a high glucosamine content and a low methionine content. This polypeptide had an estimated M_r of 83K and could only be identified in PEG precipitates of cell supernatants and not in cell lysates. The existence of a co-migrating host cell glycoprotein prevented the virus specificity of this polypeptide being confirmed. This polypeptide had N-linked oligosaccharides since it could not be detected when TRT virus infected cells were labelled in the presence of tunicamycin. The presence of other types of oligosaccharides on this protein was not ruled out by this result since the polypeptide may have been unstable when synthesized in the presence of tunicamycin. A specific antibody against this polypeptide was not available preventing further studies on this polypeptide such as pulse chase studies or identification of the size of the unglycosylated polypeptide.

TRT virus has an F-like polypeptide with disulphide bonded 45K and 15K glycosylated polypeptides. The oligosaccharides appeared to be N-linked as was observed for the PVM and RS virus F proteins (Section 3.3.7). In contrast to the situation with the PVM and subgroup A RS virus F proteins no unglycosylated form of the TRT virus 58K or 45K polypeptides could be identified when infected cells were labelled in the presence of tunicamycin. This could have been due to failure of the antiserum to recognize the unglycosylated polypeptide or instability of the unglycosylated polypeptide.

TRT virus also had a 31K glycoprotein similar to that of PVM described in Section 3.3.27. This glycoprotein was not detected by radiolabelling with [^{35}S]-methionine and produced a very diffuse band. The oligosaccharides of this polypeptide appeared to be N-linked. No equivalent polypeptide has been demonstrated in RS virus infected cells after labelling with [^3H]-glucosamine and immunoprecipitation with anti-RS virus serum. However immunoprecipitation of [^3H]-glucosamine labelled RS virus polypeptides with an antiserum raised against a synthetic peptide corresponding to the carboxy terminus of the NS1A polypeptide does precipitate a similar polypeptide (C. Caravokyri, personal communication).

The TRT virus specific 58K, 45K, 38K, 30K, 23K, 19K and 15K polypeptides were identified by immunoprecipitation of [^{35}S]-methionine labelled infected cell lysates using a murine anti-TRT virus antiserum. The 35K protein was not

identified in immunoprecipitates of [^{35}S]-methionine labelled samples but was identified in immunoprecipitates of ^{32}P labelled samples. The 35K polypeptide could be analogous to the P protein of RS virus. The 30K polypeptide has similar mobility and charge properties to the PVM and RS virus M polypeptides charge heterogeneity being observed for all three polypeptides synthesized *in vitro*. Like the PVM M protein but unlike the RS virus M protein the 30K polypeptide of TRT virus was not phosphorylated. The 19K polypeptide could sometimes be resolved into two phosphorylated bands. The two 19K polypeptides had similar properties to the PVM 20K and 19K of PVM but had no obvious counterparts in RS virus. The TRT virus polypeptides identified are compared with those observed by Collins & Gough (1988) in Table 18 and the results summarized in Table 19.

Table 18 Comparison of TRT virus polypeptides observed with
the data of Collins & Gough (1988)

Collins & Gough (1988)		Present results	
TRT virus protein	M_r ($\times 10^{-3}$)	TRT virus protein	M_r ($\times 10^{-3}$)
200K	200	-	-
-	-	129K	129
-	-	95K	95.4
84K	84	83K	82.9
54K	54	58K	58.0
42K	42	45K	45.4
37K	37	38K	38.5
31K	31	35K	35.3
		31K	31.0
		30K	30.3
		23K	22.7
		19K	19.6
14K	14	15K	15.5

Table 19 Polypeptides of TRT virus

TRT virus M_r ($\times 10^{-3}$) protein		Polypeptide identified		Acidic or basic	Identified by immuno- precipitation
		<i>in vivo</i>	<i>in vitro</i>		
129K	129	+	-	-	+
95K	95.4	+	-	-	+
83K	82.9	+	-	-	+
58K	58.0	+	-	-	+
45K	45.4	+	-	-	+
38K	38.5	+	+	acidic	+
35K	35.3	+	+	acidic	-
31K	31.0	+	-	-	+
30K	30.3	+	+	basic	+
23K	22.7	+	+	basic	+
19K	19.6	+	+	basic	+
15K	15.5	+	+	acidic	+

DISCUSSION

4 DISCUSSION

The polypeptides of RS virus, PVM and TRT virus are listed in Table 20 along with their major properties or suggested functions. In most instances the functions are based on the roles of proteins with similar properties in terms of location in the virion or nucleocapsids, charge and post-translational modifications in other paramyxoviruses or rhabdoviruses. Polypeptides with similar properties are shown in the same row of the table. The number of PVM and TRT virus encoded polypeptides has not been established since some of the less well labelled polypeptides may prove not to be virus encoded and others may be related to each other. The low titres of TRT virus stocks and the anti-TRT virus serum may have prevented the identification of poorly labelled TRT virus polypeptides. Molecular cloning of viral genes and analysis of transcripts will be required to identify the primary gene products. This may not be sufficient to identify all of the viral polypeptides present in infected cells however since some of the polypeptides detected probably arise from post-translational modifications. Monospecific antibodies, possibly raised against synthetic peptides may be required to establish the relationships between the polypeptides observed *in vivo*. The classification of PVM and TRT virus into the genus *Pneumovirus* was initially based on morphological data (Berthiaume et al., 1974; Collins et al., 1986b). The mobilities of the three major non-glycosylated polypeptides of these viruses also show some similarity (Cash et al.,

TABLE 20 Summary of pneumovirus polypeptides

Polypeptide designation			Possible function
PVM	RS virus	TRT virus	
L	L	200K	<i>RNA polymerase, transcriptase</i> ¹
G	G	83K	Attachment glycoprotein
F1	F0, F1, F2	58K, 45K, 15K	Fusion glycoprotein
N	N	38K	Nucleocapsid protein
39K	P	35K	Phosphoprotein
M	M	30K	Matrix protein
	M2		Second matrix protein
23K		22K	?
20K/19K		19K	Second phosphoprotein
18K			?
17K			?
16K			?
	NS1B		?
	NS1C		?
31K	NS1A	31K	Glycosylated protein

1) Functions/properties in italics are speculative, otherwise they are based on properties observed for the polypeptides of one or more of the viruses

1977; Collins *et al.*, 1986b). Subsequently antigenic relationships have been identified between the N and 39K polypeptides of PVM and the N and P proteins of RS virus (Gimenez *et al.*, 1984; Sections 3.1.10 and 3.2.5). Recently part of the sequence of the putative N gene of PVM has been shown to have a similar sequence to the N gene of human RS virus (J. Barr, P. Chambers & A. J. Easton, personal communication).

The characterization of the PVM and TRT virus polypeptides are discussed separately followed by suggestions for further work on these viruses.

4.1 Polypeptides of PVM

A total of 20 virus specific polypeptides were identified in experiments on PVM proteins synthesized *in vivo* and *in vitro* (Table 9). Some of these polypeptides (G1, G2, F1, N, 39K, M, 20K and 19K) have been studied in more detail than the others. In the case of these polypeptides their possible equivalence to RS virus polypeptides is discussed. The large number of polypeptides identified on gels compared with the number of genes of human RS virus suggests that some of these polypeptides are related to each other. Recent analysis of the PVM transcripts using cDNA clones suggests that PVM may have 11 genes (P. Chambers, personal communication).

The G protein of PVM appears to be processed in a different way to other viral glycoproteins. Processing of a glycoprotein to give a higher mobility form has been

observed for other viral glycoproteins for example the influenza A virus haemagglutinin (Lazarowitz et al., 1973), the gp160 glycoprotein of the human immunodeficiency virus (McCune et al., 1988) and corresponding *env* gene products of other retroviruses (e.g. Pinter & Fleissner, 1977) and the fusion proteins of the viruses in the family *Paramyxoviridae* (Sato et al., 1988; Scheid & Choppin, 1977; Fernie et al., 1985). These proteins however have disulphide bonds linking the two parts of a cleaved protein whereas experiments with the PVM G protein analysed under non-reducing conditions (Section 3.3.14) failed to detect any reduction in the intensity of the G2 polypeptide band. The HN glycoprotein of some strains of Newcastle disease virus has been observed to have a precursor (HN₀) but this protein has only N-linked oligosaccharides (Nishikawa et al., 1986). The only virus in the family *Paramyxoviridae* that has been observed to have non-N-linked oligosaccharides is RS virus which is believed to have extensive O-linked glycosylation of the G protein. A further similarity of the PVM G1 protein to the RS virus G protein is the delayed appearance of these polypeptides in pulse chase experiments. In RS virus a 45K precursor (p45) has been identified that appears to undergo further processing to give the RS virus G protein probably due to addition of O-linked sugars since this step is blocked by monensin (Appendix 1). In PVM no such precursor has been identified and monensin inhibits G1 production without any alternative form of the G protein accumulating so it is not clear if the processing of the PVM

and RS virus G proteins are similar at this stage. If the processing of the PVM and RS virus G proteins was similar the failure to detect either a precursor to G1 or a form of G1 devoid of O-linked oligosaccharides could have been due to the instability of the polypeptide or failure of the monoclonal antibody to bind to it. A 42K G related glycosylated protein was observed but there was no evidence for it being a precursor to the G1 protein or becoming relatively abundant in monensin treated cells.

The PVM G glycoprotein like the RS virus G protein does not form disulphide bonded oligomers under non-reducing conditions (Ding *et al.*, 1987; Gruber & Levine, 1983). This differs from the situation with the large (HN) glycoproteins of Sendai virus and Newcastle disease virus that form disulphide bonded dimers or trimers (Ozawa *et al.*, 1976; Hardwick & Bussell, 1979; Markwell & Fox, 1980). The large (H) glycoproteins of measles virus and CDV also form disulphide bonded dimers (Hardwick & Bussell, 1978; Lund & Salmi, 1981; Shapshak *et al.*, 1982). The possibility that the PVM G1 protein represented a dimer of the 42K protein as has been suggested with the RS virus G and p45 proteins (Fernie *et al.*, 1985) cannot be ruled out.

The difference between the G1 and G2 proteins of PVM may have been largely or even entirely due to differences in the extent of glycosylation. The G1 and G2 glycoproteins synthesized in the presence of tunicamycin or digested with glycopeptidase F showed mobility increases corresponding to reductions in M_r of 18K and 13.8K. The difference in extent

of O-linked glycosylation was even more marked, the increases in mobility of the G1t and G2t proteins following digestion with endo- α -N-acetylgalactosaminidase corresponding to reductions in M_r of 16.6K and 8.5K respectively. The M_r s of G1 and G2 synthesized in the presence of tunicamycin and digested with endo- α -N-acetylgalactosaminidase differed by only about 5K (Table 14). Since the action of endo- α -N-acetylgalactosaminidase may not remove all of the O-linked oligosaccharides there may be no difference in the size of the polypeptide of G1 and G2.

Absence of the N-linked oligosaccharides had no effect on the stability of the PVM G protein in contrast to the situation with some viral glycoproteins such as the influenza A virus haemagglutinin (Schwarz *et al.*, 1976) and the F and G glycoproteins of subgroup B strains of human RS virus (Norrby *et al.*, 1986; C. Caravokyri, personal communication). The G1 protein was also expressed at the cell surface normally in the presence of tunicamycin in contrast to the behaviour of some glycoproteins such as the measles virus fusion protein (Sato *et al.*, 1988). Processing of the G1 protein to give the G2 protein was also unaffected by the absence of N-linked oligosaccharides in contrast to, for example, the measles virus fusion protein where cleavage of the unglycosylated polypeptide does not occur (Sato *et al.*, 1988). The subtype A RS virus proteins are processed normally when synthesized in the presence of tunicamycin (Fernie *et al.*, 1985; Gruber & Levine 1985a). The F proteins of subgroup B strains of human RS virus are

unstable in the presence of tunicamycin however so the properties of glycoproteins without their N-linked carbohydrates can vary between strains of the same virus.

Processing of G1 to give G2 appeared to be a late event since monensin appeared to cause some reduction in the intensity of the G2 band relative to the G1 band and G2 appeared only after several hours of incubation in the absence of label in pulse chase experiments.

The high haemagglutination inhibition titre of the G-specific monoclonal antibody suggests that the G protein is involved in the haemagglutinating activity of the virus. Several monoclonal antibodies or monospecific antisera to each of the viral proteins would be necessary to confirm that this was the case since an antibody binding to the G protein could sterically inhibit haemagglutination involving another protein. Respiratory syncytial virus lacks a haemagglutinating activity and no such activity has been reported for TRT virus. In the *Paramyxovirus* and *Morbillivirus* genera haemagglutinating activity is associated with the larger of two viral glycoproteins so the G protein of PVM appears to be a likely candidate for the viral haemagglutinin. In all the viruses of the family *Paramyxoviridae* the large glycoprotein has been suggested to be the attachment protein. This has been demonstrated for the SV5 HN protein by Merz et al. (1981) and the RS virus G protein by Levine et al. (1987) using polyclonal sera directed against these proteins. Antibodies that inhibit attachment of the virus would be expected to neutralize the

virus. Örvell (1984) identified a group of monoclonal antibodies against one antigenic site of the mumps virus HN protein that could neutralize the virus but did not inhibit haemagglutination. Örvell & Grandien (1982) did not observe any antibodies to the Sendai virus HN protein that showed either HI or neutralizing activity independently of the other. The relationship between sites involved in haemagglutination and susceptibility of the viruses to neutralization by antibodies may therefore vary between viruses. The monoclonal antibody directed against the PVM G protein inhibited haemagglutination but did not neutralize the virus so if this protein is the viral attachment protein and the viral haemagglutinin these two functions must involve distinct sites on the protein.

The PVM F protein was not identified by Cash *et al.* (1977, 1979a) probably due to a co-migrating host cell polypeptide in their samples that were not immunoprecipitated. The expression of the F1 protein on the cell surface and its association with the G1 and G2 polypeptides in a pelleted fraction of the supernatant of PVM infected cells suggest that this polypeptide is a second major glycoprotein analogous to the F proteins of other paramyxoviruses. The RS virus F2 protein was readily labelled with [³H]-glucosamine but not with [³⁵S]-methionine and is more heavily glycosylated than the F1 protein as is the case with Sendai virus (Scheid & Chopin, 1977). In morbilliviruses only the F2 subunit appears to be glycosylated (Campbell *et al.*, 1980; Hardwick & Bussell,

1978; Mountcastle & Choppin, 1977; Örvell (1980); Tyrell & Norrby, 1978). With PVM the F1 protein was poorly glycosylated and no F2 subunit was detected by labelling with [^3H]-glucosamine, [^{35}S]-methionine or [^{14}C]-protein hydrolysate. If PVM has an F2 protein [^{35}S]-cysteine may be a suitable label to use since it would be expected to contain this amino acid. The predicted size of a PVM F2 protein is much smaller than that of the F2 proteins of other paramyxoviruses. The failure to detect an F0-like polypeptide in pulse chase experiments argues against the existence of an F2 protein. The apparent alteration in mobility of the F1 protein under non-reducing conditions could be due to internal disulphide bonding.

When a pulse chase experiment was carried out with cells labelled in the presence of tunicamycin a polypeptide with a similar mobility to F1, (designated F0t), disappeared and the F1t polypeptide appeared during the chase periods. There are three possible interpretations of this result. The disappearance of F0t and appearance of F1t may have been coincidental although this seems unlikely. F0t may have represented an unglycosylated form of F0 that was detected in the absence of glycosylation due to slower processing. Finally, the F0t polypeptide may have represented F1 that was sensitive to, for example, protease activity giving rise to the higher mobility F1t band during chase periods. A lack of N-linked carbohydrate might account for the instability of the F0t polypeptide. Although F1 and F0t had similar mobilities the extent of glycosylation of F1 appeared to be

low so this might be expected. This is supported by the failure to observe any mobility change when F1 was digested with glycopeptidase F. Increases in the mobility of F1 synthesized in the presence of tunicamycin could also have been due to altered cleavage of a signal peptide in the absence of glycosylation or altered translocation leaving part of the protein on the cytoplasmic side of the membrane that could subsequently be removed by proteases. The nature of the F protein of PVM is therefore unclear but it appears to differ from that of other viruses in the family *Paramyxoviridae* in that it has a very small F1 subunit or none at all and it is very poorly glycosylated.

The 48K polypeptide synthesized *in vitro* was not observed in immunoprecipitates of PVM infected cell lysates or PEG precipitates of the supernatants of PVM infected cells. It was possible that this polypeptide represented an unglycosylated form of one of the glycoproteins. It was too large to represent an unglycosylated form of the PVM G protein. This was the only potential glycoprotein identified by labelling *in vitro* which favours the possibility that it was related to the F protein of PVM since this protein was more readily labelled with amino acid labels than the G protein. Labelling of the 48K polypeptide to a higher specific activity followed by a comparison of the tryptic peptide map of this polypeptide to those of PVM polypeptide synthesized *in vivo* would probably be the best way of resolving this difficulty.

The N polypeptide of PVM was identified as the

nucleocapsid polypeptide by Cash et al. (1979a). Either the N or the 39K polypeptide was phosphorylated. The nucleocapsid proteins of canine distemper virus (Hall et al., 1980), measles virus (Robbins & Bussell, 1979), mumps virus (Rima et al., 1980) and Sendai virus (Lamb & Choppin, 1977) are phosphorylated. The N protein of Sendai virus also exists in a non-phosphorylated form (Lamb & Choppin, 1977) and the nucleocapsid protein of RS virus is not phosphorylated. There are therefore precedents for both phosphorylated and non-phosphorylated nucleocapsid proteins. The N protein of PVM shows a similar streak when identified by two dimensional NEPHGE/SDS-PAGE to the N protein of RS virus (Figure 7) suggesting that it may have similar properties to the N protein of RS virus.

The 39K polypeptide of PVM may be analogous to the RS virus P protein. The 39K polypeptide may represent the phosphorylated polypeptide, the 35K polypeptide which gives a similar peptide profile to the 39K protein after digestion with chymotrypsin (Figure 7) representing an unphosphorylated form of the protein. In Sendai virus the P protein has been observed to be relatively more abundant in infected cells than in virions (Lamb et al., 1976; Portner & Kingsbury, 1976). The 39K polypeptide was observed to be more readily immunoprecipitated from cell lysates than from dissociated PEG precipitates of culture supernatants suggesting that the 39K polypeptide is also more abundant in infected cells than in the culture supernatants. The

unphosphorylated 35K polypeptide was only detected in cell lysates and has not been identified in partially purified virus (Cash et al., 1979a). There is no evidence of a progression of label from the 35K polypeptide to the 39K polypeptide during pulse chase experiments. This suggests that either no interchange between the forms of the 39K protein occurs or that an equilibrium is reached with only the 39K form being incorporated into virions. The significance of the 35K polypeptide is not clear since it has not been detected in lysates of PVM infected BHK-21 cells in which PVM is able to multiply (Cash et al., 1979a). The cross reaction of the anti-39K polypeptide monoclonal antibody 26/4/C4 with the RS virus P protein provides further evidence that these polypeptides may have similar functions. Immunoprecipitation of 32 phosphorous labelled PVM proteins with the anti-39K polypeptide 26/4/C4 would determine if the 39K polypeptide was in fact phosphorylated.

The M protein of PVM has not been shown to be envelope associated since insufficient quantities of radiolabelled virus could be obtained. The M proteins of PVM and RS virus do however share a similar basic nature. On two dimensional NEPHGE/SDS-PAGE gels minor spots with the same mobility as the M protein were observed with both PVM and RS virus polypeptides. The spots may represent conformational variation of the M protein such as different disulphide bond arrangements. Different forms of M have been observed under non-reducing conditions with both PVM (data not shown) and RS virus (Gruber & Levine, 1983). Although the NEPHGE sample buffer contained 2-mercaptoethanol it did not contain SDS

and the samples were not heated so not all of the disulphide bonds may have been disrupted. The matrix proteins of Sendai virus and measles virus have an affinity for lipid and nucleocapsids suggesting that they may be involved in assembly of the virus (Shimizu & Ishida, 1975; Tyrell et al., 1980; Yoshida et al., 1976). As described in the introduction the RS virus matrix protein remains associated with subviral particles. The M protein of PVM was co-precipitated with the 39K polypeptide suggesting that these polypeptides may be associated.

There was some evidence that the 20K and 19K polypeptides may have been related but this was only from a partial proteolysis experiment and the 20K and 19K polypeptides may not have been sufficiently well resolved to excise the bands cleanly. One or both of these polypeptides was phosphorylated. If the polypeptides are related the mobility difference between them could have been due to different degrees of phosphorylation. This might also account for differences in the intensity of the 20K and 19K polypeptides observed *in vitro*. There was no obvious counterpart of the 20K or 19K polypeptides in RS virus although an 18K phosphorylated polypeptide of unknown origin was identified. The 20K and 19K polypeptides were readily detected in PEG precipitates of PVM infected cells suggesting that they were probably structural polypeptides of the virus.

Little is known about the L, 25K, 24K, 23K, 18K, 17K, 16K, and 12K PVM polypeptides or the 31K glycosylated

polypeptide. Since the 18K and 17K polypeptides were only regularly identified when synthesized *in vitro* they probably represent primary translation products that are modified to give other proteins in infected cells. A 17K polypeptide was in fact identified in infected cells after a pulse labelling period (Section 3.3.20). The 12K protein was not a primary gene product appearing only after a chase period (Section 3.3.20).

4.2 TRT virus polypeptides

The TRT virus polypeptides identified are summarised in Table 19 and compared with those described by Collins & Gough (1988) in Table 18 and with those of the pneumoviruses in Table 20. The 200K polypeptide described by Collins & Gough (1988) in preparations of partially purified virus was not observed probably due to low levels of label incorporation. A 200K polypeptide was immunoprecipitated by a chicken anti-TRT virus serum but the result was difficult to interpret since the only other polypeptides immunoprecipitated by this serum were the 38K TRT virus polypeptide and the RS virus N protein. All of the other polypeptides described by Collins & Gough (1988) were observed in infected cell lysates or PEG precipitates of culture supernatants.

The 83K glycoprotein was not observed in cell lysates suggesting that it may be derived from an intracellular precursor of different mobility. The 95K glycoprotein observed only in cell lysates was a possible candidate for a

precursor of the 83K polypeptide. Pulse chase experiments would be required to determine if the 95K and 83K polypeptide did show such a relationship. Ideally an amino acid label would be used along with immunoprecipitation by a specific antibody to carry out such an experiment. If the protein was very difficult to label with amino acid labels [³H]-glucosamine could probably be used because although label incorporation continues during the chase period the appearance of the PVM G2 protein after a chase period could be detected in this way. Labelling of TRT virus infected cell with [³H]-glucosamine in the presence of tunicamycin showed that all the TRT virus glycoproteins had N-linked oligosaccharides. This did not exclude the possibility that other types of oligosaccharides occurred since the glycoproteins devoid of N-linked oligosaccharides may have been unstable. This could be examined further by digesting the glycoprotein bands excised from gels with glycosidases. The label incorporation achieved with TRT virus polypeptides was low compared with the label incorporation into PVM polypeptides. To avoid the losses of label observed during digestion of PVM and RS virus glycoproteins it would probably be better to digest the glycoproteins in the gel slices after washing them in a similar way to that described for the tryptic peptide mapping experiments. The gel slice and any fluid could then be boiled with sample buffer and loaded into the gel slots avoiding any significant loss of labelled glycoprotein. The nature of the 95K and 83K polypeptides if they represent distinct proteins is not

clear although either or both proteins could represent aggregates of lower molecular weight polypeptides. The function of the 83K polypeptide is not known, no haemagglutinating or neuraminidase activity has so far been demonstrated for TRT virus. By analogy with the G protein of RS virus which has a similar mobility it could be the attachment protein of the virus.

The 45K and 15K glycosylated polypeptides had similar properties to the F1 and F2 polypeptides of RS virus. Under non-reducing conditions these bands were reduced in intensity and a band of 58K was detected. The most likely explanation of this is that the 45K and 15K polypeptides are disulphide bonded. To demonstrate that they are derived by cleavage of a precursor polypeptide a pulse chase experiment would need to be carried out to show the passage of labelled amino acid from a precursor of about 58K to the 45K and 15K polypeptides. An attempt to demonstrate this was unsuccessful due to inadequate label incorporation into pulse labelled polypeptides immunoprecipitated with the anti-TRT virus serum.

The 38K polypeptide was one of the two most readily detected TRT virus polypeptides. Its mobility was similar to that of the nucleocapsid proteins of PVM and RS virus and it was acidic although it did not show such pronounced streaking during NEPHGE as the nucleocapsid proteins of PVM and RS virus. Like the nucleocapsid protein of RS virus it does not appear to be phosphorylated. It was present in material isolated using a method designed for the isolation of RS

virus nucleocapsids (Ward et al. 1983) and which gave material containing predominantly the N polypeptide of PVM when used with lysates of PVM infected cells. However, the 35K and 30K polypeptides were also present in the material isolated from TRT virus infected cells (data not shown).

The 35K polypeptide of TRT virus had a similar mobility and charge to the P protein of RS virus and the 35K polypeptide of PVM. Like the P protein of RS virus the 35K polypeptide of TRT virus is phosphorylated. The 35K polypeptide of PVM is not phosphorylated but as discussed above is related to the 39K polypeptide that may be phosphorylated. The 35K polypeptide synthesized *in vitro* is indistinguishable on the basis of mobility and charge from the 35K polypeptide synthesized *in vivo* suggesting that it may be phosphorylated *in vivo*.

The 30K polypeptide of TRT virus has been identified in radiolabelled cell lysates, amongst the products of *in vitro* experiments and on stained gels of partially purified virus (Collins & Gough, 1988; Ling & Pringle, 1988). It is readily detected and appears to be basic like the M proteins of PVM and RS virus. The 30K polypeptide co-migrates with several minor spots revealed on two dimensional gels. The different spots could represent polypeptides with different arrangements of disulphide bonds, two dimensional SDS-PAGE using non-reducing conditions for the first dimension and reducing conditions for the second dimension would enable this to be established. The 30K polypeptide of TRT virus migrates with a lower mobility than the PVM and RS virus M

proteins. The relative position and intensity of the 30K polypeptide band does however resemble that of the PVM and RS virus M proteins. It seems likely that the 30K polypeptide of TRT virus is a matrix-like protein.

The 23K and 19K polypeptides of TRT virus were not observed in preparations of partially purified virus by Collins & Gough (1988) and may be non-structural polypeptides. The 19K polypeptide could however be identified in PEG precipitates of culture supernatants. The 23K polypeptide was however basic like the RS virus M2 protein and resembling this protein in its location on two dimensional gels more than any other pneumovirus polypeptide. The 19K polypeptide did not clearly resemble any pneumovirus polypeptide being phosphorylated and having a similar mobility to the PVM 20K and 19K polypeptides but being basic when identified on two dimensional gels.

4.3 Suggestions for further work

There are several experiments that could be performed to further characterize the polypeptides of PVM, TRT virus and RS virus. Digestion with glycosidases could be used to determine the extent of N-linked and O-linked glycosylation on the viral glycoproteins directly. This would be most informative for the TRT virus glycoproteins where all that is known is that some N-linked oligosaccharides are present on the glycoproteins. The digestion of the G1t and G2t polypeptides of PVM with endo- α -N-acetylgalactosaminidase could usefully be repeated since it is not known how

reproducible or complete this digestion is. The use of sugar labelling and/or different enzyme concentrations would give an indication of whether all the carbohydrate is removed. The use of G1t and G2t rather than G1 and G2 would remove the complication of the digestion of N-linked carbohydrate by the exoglycosidases or the requirement to obtain conditions under which the proteins could be digested by both glycopeptidase F and endo- α -N-acetylgalactosaminidase. Digestion with both enzymes would be required to study the TRT virus glycoproteins because they appear to be unstable when synthesized in the presence of tunicamycin. Digestion of the RS virus G protein synthesized in the presence of tunicamycin would be useful to give a direct indication of the degree of O-linked glycosylation on this protein. Pulse chase experiments to determine the relationships between TRT virus glycoproteins have already been mentioned. Studies on the non-glycosylated proteins mainly require the resolution of closely migrating bands such as the 20K and 19K polypeptides and increased label incorporation in the minor polypeptides such as the various bands observed in the 20-25K region of the gel and the polypeptides with apparent M_r s of less than 19K. These polypeptides need to be studied by peptide mapping along with the major viral polypeptides to determine which of them represent unique viral proteins and which of them are related to other viral proteins. Radiolabelling of polypeptide bands with iodine -125 would enable higher specific activities to be obtained for tryptic peptide mapping. This approach was not attempted in the

experiments described in Section 3.3 because of the immunoglobulin bands of 45-50K and 22-24K regions of the gel. This problem could be overcome by using a two dimensional gel with a non-reducing first dimension that would separate the immunoglobulin, migrating as an intact 150K molecule under these conditions), from the viral polypeptides. The viral polypeptides could then be identified from their mobilities on the dimension gel. Alternative methods of preparing TRT virus nucleocapsids and detergent and salt treatment of partially purified virus could be used to determine the location of the viral polypeptides in virions. The possibility of determining whether the N or 39K polypeptide of PVM was phosphorylated by immunoprecipitation with the anti-39K protein monoclonal antibody 26/4/C4 has already been mentioned. An interesting observation was the low background of cellular polypeptides observed when polypeptides were synthesized *in vitro* using RNA from infected cells treated with actinomycin D. This could have been due to the RNA being synthesized for an extended period in the presence of actinomycin D prior to labelling of polypeptides. Actinomycin D apparently had no effect on the background of cellular polypeptides synthesized in infected cells but labelling in this case was begun only one hour after treatment with actinomycin D. The effect of treatment with actinomycin D 12 to 24 hours prior to labelling for 1 hour might reveal if timing of the labelling would explain the discrepancy between the results obtained *in vitro* and *in vivo*. Another explanation might be that a protein bound to

the viral mRNA molecules and inhibited translation which could then take place after the RNA had been extracted and added to an *in vitro* translation system. This possibility could easily be tested by adding extracts of uninfected and PVM infected cells to *in vitro* translation reactions. This would reveal whether translation of the viral mRNA was inhibited by a cellular or a viral protein.

The⁶ results presented in this thesis describe the first detailed study of PVM glycoproteins and a preliminary study of the TRT virus proteins. Further characterization of these viruses awaits molecular cloning of the viral genomes and production of additional specific antibodies to the viral proteins. Some progress on molecular cloning of these viruses has already been made in the same laboratory by P. Chambers, J. Barr and A. J. Easton, the PVM clones being prepared from the cellular RNA extracted from PVM infected cells that was used in the *in vitro* translation experiments described in Sections 3.1.7-9 and 3.5.4. The characterization of the viral genomes will show the number of primary gene products and enable the relationship of these viruses to RS virus to be more clearly established. cDNA clones will enable studies of both transcription of viral genes and identification of polypeptides synthesized *in vitro* to be carried out. In addition sequence data would enable synthetic peptides to be produced. Such peptides would provide convenient antigens for raising antisera for studying polypeptide synthesis *in vivo*. cDNA clones and specific antibodies would be useful for attempts to identify

any PVM related viruses in clinical samples from human patients. Further isolates of PVM would be required to establish if significant strain variation occurs.

APPENDICES

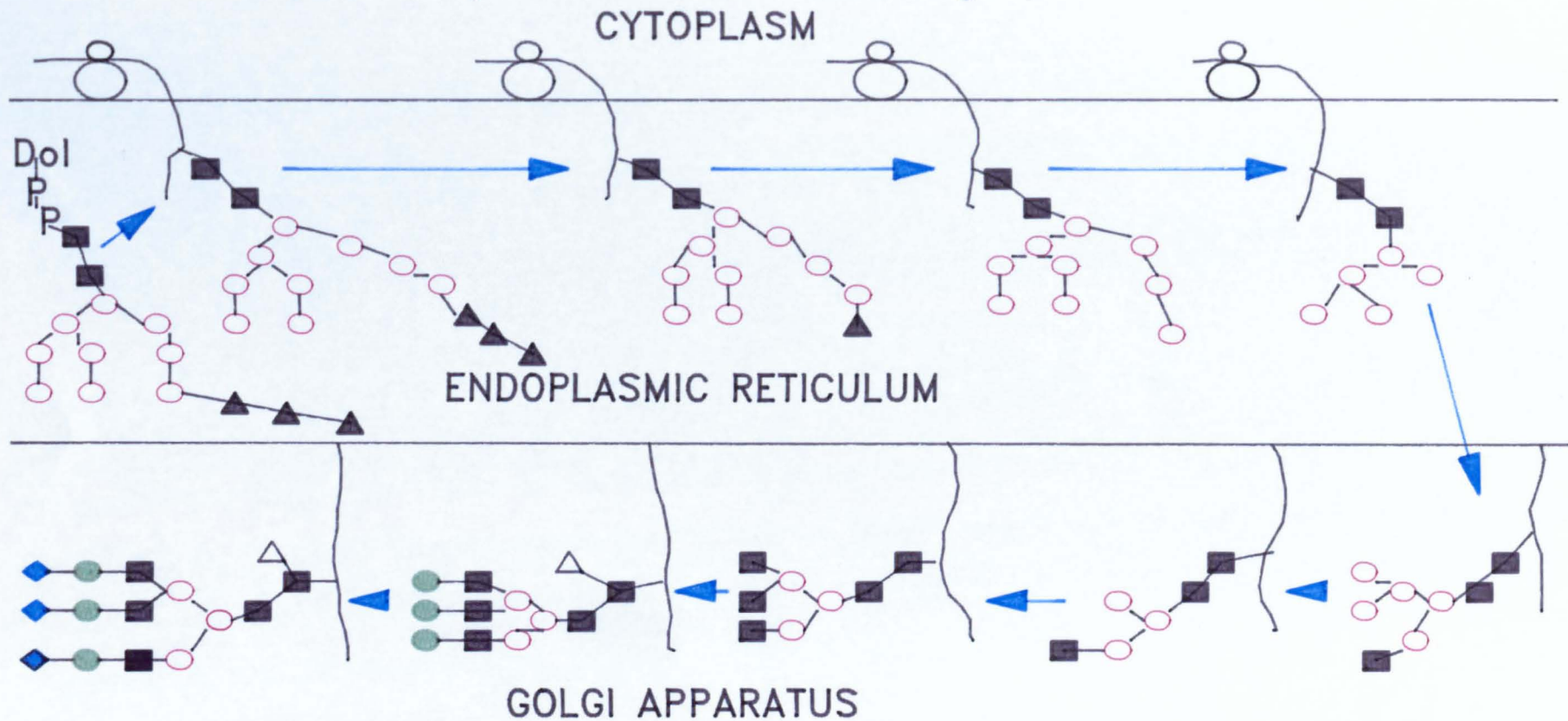
APPENDIX 1

Glycosylation of polypeptides

Two types of oligosaccharides occur in glycoproteins, distinguished by the nature of the linkage to the polypeptide chain. Many oligosaccharides are linked to asparagine residues and are described as being N-linked. Other oligosaccharides may be linked to serine, threonine or less commonly hydroxylysine or hydroxyproline residues. These are referred to as O-linked oligosaccharides.

N-linked oligosaccharides are initially synthesized and added to the polypeptide in the rough endoplasmic reticulum (Katz *et al.*, 1977; Rothman & Lodish, 1977; Sefton, 1977; Tonezuzzo & Ghosh, 1977, 1978). N-acetylglucosamine is transferred from N-acetylglucosamine-UDP to dolichol phosphate followed by the transfer of a further N-acetylglucosamine residue, 9 mannose residues and 3 glucose residues from glycosyl-nucleoside diphosphates to the oligosaccharide chain (reviewed by Kornberg & Kornberg, 1980; Waechter & Lennarz, 1976). Figure 69 shows a diagram of the synthesis and processing of N-linked carbohydrates. This precursor oligosaccharide is then transferred to an asparagine residue of a newly synthesized polypeptide chain at the sequence Asn-X-Ser(Thr) where X can be any amino acid. This sequence is required for attachment of the oligosaccharides but not every sequence of this type is glycosylated. The precursor oligosaccharide is processed during transport to the Golgi apparatus by removal of the glucose and a variable number of

FIGURE 69 Synthesis of N-linked oligosaccharides



KEY:-

Dol Dolichol
P Phosphate

■ N-Acetylglucosamine

○ Mannose
▲ Glucose
● Galactose

◆ Neuraminic acid
△ Fucose
○ Ribosome

the mannose residues to give a high mannose oligosaccharide. The oligosaccharide may then be further processed by the addition of N-acetylglucosamine, galactose, neuraminic acid and fucose residues within the Golgi apparatus to give a complex form. The involvement of the Golgi apparatus in processing of complex carbohydrates has been indicated by the enrichment of the glycosyltransferases in fractions enriched in this organelle (Munro et al., 1975) and by autoradiography (Haddad et al., 1971). A single glycoprotein may have both high mannose and complex carbohydrates, for example thyroglobulin (Arima & Spiro, 1972) and IgM (Hickman et al., 1972; Shimizu et al., 1971). A single glycoprotein may also possess both complex and O-linked oligosaccharides, for example fetuin (Spiro, 1973) and human IgA (Baenziger & Kornfield, 1974a and b).

Tunicamycin is an antibiotic that inhibits transfer of sugars to dolichol phosphate and as a result inhibits addition of N-linked oligosaccharides to polypeptides (Waechter & Lennarz, 1976). It does not inhibit attachment of O-linked oligosaccharides to glycoproteins (Holmes et al., 1981; Shida & Dales, 1981). Endo- β -N-acetylglucosaminidase H cleaves between the two N-acetylglucosamine residues proximal to the polypeptide chain in high mannose but not complex carbohydrates (Arakawa & Muramatsu, 1974; Tarentino et al., 1974). Endo- β -N-acetylglucosaminidase D catalyses a similar cleavage with a specificity for complex oligosaccharides without side chains and with a Man₃GlcNAc₂-Asn sequence or

complex oligosaccharides with one side chain and one terminal mannose residue (Ito et al., 1975). Endo- β -N-acetylglucosaminidase F (Endo F) cleaves N-linked oligosaccharides without specificity for the nature of the carbohydrate. Glycopeptidase F is similar to Endo F but cleaves between the asparagine and the first glucosamine residue. These enzymes have been used to determine the nature of N-linked oligosaccharides of glycoproteins.

O-linked oligosaccharides are synthesized in the Golgi apparatus by transfer of individual oligosaccharides from glycosyl-nucleoside diphosphates to serine or threonine residues or other sugars attached to such residues. They vary in complexity from simple disaccharides in submaxillary mucins to complex branched structures such as those of blood group antigens (reviewed by Kornfield and Kornfield, 1980). No common amino acid sequences have been identified around glycosylated serine/threonine residues and it has been suggested that acceptor regions have little secondary structure making them accessible to the UDP-N-acetylgalactosamine:polypeptide α -N-acetyl-galactosaminyl transferase (Hill et al., 1977). Serine and threonine linked oligosaccharides are normally identified by their susceptibility to removal by alkaline borohydride treatment but this procedure results in destruction of the polypeptide chain and so cannot be used for studying the polypeptide. Other O-linked oligosaccharides are attached to hydroxyproline or hydroxyproline but these appear to be restricted to limited types of glycoproteins. Hydroxylysine

attached oligosaccharides occur in collagens (Morgan *et al.*, 1970) and hydroxyproline attached oligosaccharides occur in proteins in plant cell walls known as extensins (Lamport *et al.*, 1973). Another type of O-linked oligosaccharide has been described in which single N-acetylglucosamine residues are attached to the polypeptide and in which the monosaccharides are added in the cytoplasm (Davis & Blobel, 1987). Only one enzyme specific for O-linked oligosaccharides appears to be available. Endo- α -N-acetylgalactosaminidase cleaves unsubstituted galactose disaccharides from serine and threonine residues but does not work if either galactose residue is substituted so additional sugars need to be removed with exoglycosidases. Monensin, an ionophore specific for monovalent cations, has been used as an inhibitor of Golgi apparatus functions including addition of O-linked oligosaccharides to polypeptides (Alonso & Companys, 1981; Johnson & Schlesinger, 1980; Johnson & Spear, 1982, 1983; Niemann *et al.*, 1982; Pressman 1976).

Tunicamycin, monensin, Endo H, Endo D and Endo F have been used in the study of RS virus glycoproteins.

APPENDIX 2

Suppliers names and addresses

Amersham International plc, Aylesbury, Bucks., UK

Amplify

Biotinylated anti-mouse Ig, general purpose screening reagent

Biotinylated peroxidase plus streptavidin

Biotinylated protein A

D-[1-³H]-glucosamine hydrochloride 5.8Ci mmol⁻¹

L-[4,5-³H]-leucine

L-[³⁵S]-methionine 1490Ci mmol⁻¹

Rabbit reticulocyte lysate

Streptavidin-biotinylated peroxidase complex

Bantin & Kingman Ltd, Grimston, Hull, UK

Balb/c mice, female 6-8 weeks old

BDH Chemicals Ltd, Atherstone, Warwks., UK

Citric acid

Glutaraldehyde solution (50%)

2-Mercaptoethanol

Nonidet P-40

Orthophosphoric acid

Phenol

Polyethylene glycol 1500

Polyethylene glycol 6000

Potassium chloride

Potassium dihydrogen orthophosphate
Sodium hydroxide
di-Sodium hydrogen orthophosphate

Beckman RIIC Ltd., High Wycombe, UK

Ready-solv EP liquid scintillation fluid

Bio-rad Laboratories Ltd., Watford, Herts., UK

HRP colour development reagent

Trans blot apparatus

Boehringer-Mannheim (BCL), The Boehringer Corporation Ltd,
Lewes, East Sussex, UK

Polyethylene glycol (50% solution in HEPES)

Tunicamycin

Glycopeptidase F

Endo- α -N-acetylgalactosaminidase

BRL (Bethesda Research Laboratories) Ltd., Renfrew Road,
Paisley, UK

Ammonium persulphate

Carbon-14 labelled molecular weight markers

Immunoprecipitin

Chance Propper Ltd., Smethwick, Warley, UK

Cover glasses, 17mm diameter, No.1.5

Microscope slides, 76X26X1.0-1.2mm

Fisons PLC (Services), Crawley, Sussex, UK

Acrylamide, specially purified for electrophoresis

Ammonium hydrogen carbonate

Butan-1-ol

Calcium chloride

Chloroform

Diaminoethanetetra-acetic acid, disodium salt

Diethyl ether

Dimethyl sulphoxide

2,5-Diphenyloxazole

Glycerol

Glycine

Hydrogen peroxide (100 vols.)

Lithium chloride

Magnesium chloride

Magnesium sulphate

Nitric acid

Sodium chloride

Sodium lauryl sulphate, specially purified for
biochemical work

Trichloroacetic acid

Urea

Flow Laboratories Inc., Rickmansworth, Herts., UK

Foetal calf serum

Glasgow modification of Eagles medium

HLA plates, 60 well

Round bottomed 96 well plates

Fuji Photo film Co. (UK) Ltd., 125 Finchley Road, London, UK

RX medical X-ray film

Gibco Europe Ltd., Paisley, Scotland

Foetal calf serum

Glutamine (200mM)

Leucine (200mM)

Methionine (200mM)

Non-essential amino acids (100X)

Tryptose phosphate broth

Valine (200mM)

Kodak Chemicals Ltd., Kirby, Liverpool, UK

N,N-methylene bis-acrylamide

LKB Instruments Ltd., Milton Keynes, Bucks., UK

Ampholines pH3.5-10

Ampholines pH5-7

May and Baker Ltd., Eccles, Manchester, UK

Acetic acid, (glacial)

Ethanol

Hydrochloric acid

Methanol

Miles Laboratory Ltd., Stoke Poges, Slough, UK

Mixed glycosidases from *Turbo cornutus*

Nordic Immunological Reagents Ltd., Maidenhead, Berks., UK

Rabbit anti-mouse immunoglobulins-FITC conjugate

Northumbria Biologicals Ltd., Northumberland, UK

Costar 6, 12, and 24 well tissue culture clusters

Costar 25cm², 50cm² and 150cm² tissue culture flasks

ESG hybridoma growth factor

Schleicher and Schuell, Dassel, West Germany

BA85 membrane filter

Cellulose thin layer chromatography plates (20X20cm)

Sigma chemical Company, Poole, Dorset, UK

Acid fuchsin

Actinomycin D

Aminopterin

Bovine serum albumin

Crystal violet

Diethyl pyrocarbonate

Freunds complete adjuvant

Freunds incomplete adjuvant

HAT medium supplement

HEPES
HT medium supplement
Hypoxanthine
L-methionine
Monensin
Neuraminidase
Orange G
Orthophenylenediamine
Paraformaldehyde
Phenylmethylsulphonylfluoride
Polyoxyethylene sorbitan monolaurate (Tween 20)
Ponceau S
2,6,10,14-Tetramethylpentadecane
Thymidine
Triton X-100
Trizma base
V8 protease

Sera lab, Crawley Down, Sussex, UK

Foetal calf serum

Sterilin, Teddington, Middlesex, UK

96 well tissue culture plates

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